REMARKS

I.   Introductory Remarks

Applicants request reconsideration of this application in view of the foregoing amendments and the following remarks.

Upon entry of the amendments, claims 1-26 and 32-33 will remain pending in the application, with claims 6-10 and 15-26 being withdrawn from consideration. Claims 1-2, 11-14 and 32-33 are currently being amended. No claims presently are being canceled or added.

The claim amendments correct typographical errors, make the claim language better correspond between independent and dependent claims and clarify that the treatment method pertains to *human* papillomavirus infections. Exemplary support for substitution of the word 'transformed' into claim 33 exists in the fourth full paragraph on page 5 of the specification. None of the amendments introduce new matter into the application.

II.   Claims 1-5 are Patentable over U.S. Patent 6,436,402

The Office rejected claims 1-5 as being anticipated under 35 U.S.C. § 102(e) by U.S. Patent 6,436,402 ("the '402 patent"). Applicants respectfully traverse this rejection.

The '402 patent does not constitute prior art, as defined by 35 U.S.C. § 102(e). The present application has a priority date of December 11, 1998, the filing date of Australian application PP 7653. In comparison, the earliest possible 102(e) date for the '402 patent is October 15, 1999. Thus, the present application predates the '402 patent by at least 10 months.

Because the '402 patent is not prior art, Applicants request withdrawal of the rejection.

III.   Claims 1-5, 11-14 and 32-33 are Patentable over the Work of Kirnbauer *et al.*

The Office rejected claims 1-5, 11-14 and 32-33 under 35 U.S.C. § 102(b)/103(a) as being anticipated by or obvious over Kirnbauer *et al.*, J. Virol. 67(12): 6929-36 (1993) ("Kirnbauer 1993"). Applicants respectfully traverse this rejection.

Kirnbauer 1993 neither teaches nor suggests a method of *treating* existing human papillomavirus infections. Kirnbauer 1993 reported the efficient assembly of HPV16 VLPs and noted that the ability to produce such VLPs "may have implications for . . . *immunoprophylaxis* against HPV16 infection." (Kirnbauer 1993 abstract, emphasis added) The complete discussion of vaccines in Kirnbauer 1993 essentially exists in the last paragraph of that publication:

> Efficient self-assembly of HPV16 particles may also have implications for
> efforts to develop an effective *prophylactic vaccine* against HPV16 infection.
> In previous vaccination experiments, bacterially expressed capsid proteins
> were partially effective in *preventing experimental infection* in animals,
> although they induced only low levels of neutralizing antibodies (9, 11, 15).
> We recently reported that immunization of rabbits with native, but not
> denatured, VLP from BPV L1 induced high-titer neutralizing antibodies (10).
> This observation suggests that VLP might have the potential to *induce long-*
> *lasting immunity*, a characteristic that might be advantageous for an effective
> HPV vaccine.

(Kirnbauer 1993, ¶ bridging col. 1-2 on p. 6935, emphasis added). Each reference to a vaccine in Kirnbauer 1993 specifically pertains to *prophylactic administration*. Thus, the reference does not anticipate the rejected claims, which are directed to "treatment of an existing human papillomavirus (HPV) infection," and not to the prevention, or prophylaxis, of future infections.

Kirnbauer 1993 also fails to provide any suggestion or motivation for administering L1- or L1/L2-containing HPV VLPs to treat existing infections. As noted above, all of that publication's references to vaccines relate to prophylactic, and not therapeutic vaccines. Moreover, skilled artisans would not have expected HPV L1- or L1/L2-containing VLPs to be useful for treating existing infections because the L1 and L2 proteins are undetectable in the basal epithelial cells of HPV lesions. Instead, as of the priority date of the current application, skilled artisans believed that a therapeutic vaccine should comprise HPV early proteins, or E proteins. Numerous contemporary publications evidence this prevailing belief:

> Greenstone *et al.*, PNAS, USA 95: 1800-1805, 1800 (February 1998): "It is
> unlikely that cell-mediated responses to L1 or L1/L2 VLPs would have a
> significant therapeutic effect against established papillomaviral infections.
> This speculation is based on the observation that L1 and L2 proteins are

undetectable in the most likely targets of immune regression: the basal epithelial cells of benign productive lesions and the abnormal proliferative cells in premalignant and malignant lesions (13). In contrast, other viral genes, such as E7 or E2, are likely to be expressed in these cells. Therefore, these proteins are potential targets for cell-mediated immune regression."

Schiller and Roden, Papillomavirus Report 6(5): 121-128, 124 (1995): "It is unlikely that an L1 or L1/L2 VLP vaccine will be effective in eliminating HPV induced lesions, since the late genes are not detectably expressed in progressed lesions or the replicating basal cells of benign lesions. Incorporation of early proteins into the VLPs might increase the therapeutic potential of a VLP-based vaccine."

WO 98/10790, p. 3: "For the vaccine to be therapeutic, it must elicit a cell-mediated immune response targeted toward the specific HPV antigens; such a response will kill HPV-infected cells, thereby preventing disease progression. The induction of the cell-mediated immunity is accomplished via the injection of viral E proteins, which must be taken up by the cell, processed, and presented complexed with the appropriate MHC Class I molecules by antigen-presenting cells. Thus, the prophylactic/therapeutic HPV vaccine will consist of VLPs in combination with the appropriate HPV E proteins."

WO 99/18220, p. 3: "Since the L1 protein is not present in malignant genital lesions, vaccination with L1 protein does not have any therapeutic potential for these patients. Construction of chimeric proteins, comprising amino acid residues from L1 protein and, for example E6 or E7 protein, which give rise to chimeric capsomeres, combines prophylactic and therapeutic functions of a vaccine."

Hagensee, Infections in Medicine 14(7): 555-564, 560 (1997): "*Therapeutic vaccine targets.* There are many potential antigenic targets for a therapeutic vaccine. The replication proteins E1 and E2 may be required for

establishment of a genital infection. The E4 and E5 transcripts are the most abundant, making them possible targets, although the function of these proteins is not known. E6 and E7 are being considered for a therapeutic cervical cancer vaccine since they are expressed during the late stages of disease. Finally, L1 and L2 are not always present in late stages of disease, making their use as potential therapeutic targets still plausible but with limitations."

See also Hines et al., Curr. Opin. Infect. Dis. 11: 57-61 (1998).

Against this overwhelming scientific consensus that a therapeutic vaccine would require HPV E protein, Applicants surprisingly discovered that vaccines without HPV E protein are effective therapeutic vaccines. For this reason, the rejected claims are patentable over Kirnbauer 1993, and Applicants request withdrawal of the rejection.

IV.   The Claims are Patentable over U.S. Patent 5,888,516

The Office rejected claims 1-5, 11-14 and 32-33 under 35 U.S.C. § 102(e)/103(a) as being anticipated by or obvious over U.S. Patent 5,888,516 ("the '516 patent"). Applicants respectfully traverse this rejection.

The '516 reference is not an enabling prior art reference and does not provide a reasonable expectation of success for treating existing HPV infections. The '516 patent "is particularly directed to the production of a *prophylactic* vaccine for papillomavirus infection." ('516 patent, col. 5, ll. 18-20, emphasis added). Vaccines described in the '516 patent comprise HPV VLPs that consist of the L1 and/or L2 proteins, like the VLPs of Kirnbauer 1993.

The '516 patent makes a passing reference to therapeutic vaccines, stating that "[t]he present invention is directed to the production of a prophylactic and *possibly* therapeutic vaccine for papillomavirus infection" ('516 patent, col. 2, ll. 56-58, emphasis added), but focuses strictly on prophylactic vaccines. Because it contains no actual teachings regarding therapeutic vaccines and those and a skilled artisan would have believed that a therapeutic

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vaccine must contain an HPV E protein (see section III above), the '516 patent does not enable the present application's claims.

It is well settled that prior art under 35 U.S.C. § 102 must be enabling, meaning that it places the public in possession of the claimed invention. *In re Donahue*, 766 F.2d 531 (Fed. Cir. 1985). "Tossing out the mere germ of an idea does not constitute enabling disclosure." *Genentech, Inc. v. Novo Nordisk A/S*, 108 F.3d 1361, 1366 (Fed. Cir. 1997). Yet, the '516 patent does no more than toss out the mere germ of an idea for therapeutic vaccines. In an art that was undeveloped and highly unpredictable, the '516 patent provided no direction or guidance for treating existing HPV infections and contained no working examples of doing so. For these reasons, the '516 patent is not an enabling prior art reference and therefore does not anticipate the rejected claims.

Additionally, the '516 patent did not create a likelihood of success for treating existing HPV infections. As described in detail above, skilled artisans would not have expected HPV L1- or L1/L2-containing VLPs to be useful for treating existing infections because the L1 and L2 proteins are undetectable in the basal epithelial cells of HPV lesions. Rather, artisans believed that a therapeutic vaccine should comprise papillomavirus E proteins. For these reasons, the '516 patent does not render the claimed invention obvious.

Because the '516 patent neither anticipates nor renders obvious the claimed invention, Applicants request withdrawal of the rejection.

V.   Concluding Remarks

Applicants request prompt and favorable reconsideration of this application, which is now in condition for allowance. If the Examiner believes that an interview would advance prosecution, he is invited to contact the undersigned by telephone.

The Commissioner is hereby authorized to charge any additional fees that may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to

Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. § 1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.
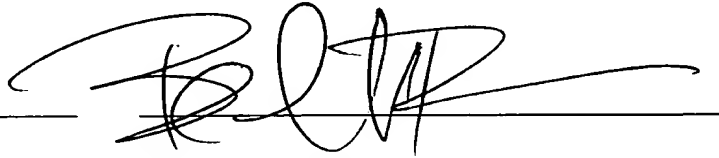
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PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 39/12	**A1**

(11) International Publication Number: **WO 98/10790**

(43) International Publication Date: 19 March 1998 (19.03.98)

(21) International Application Number: PCT/US97/15820

(22) International Filing Date: 8 September 1997 (08.09.97)

(30) Priority Data:
60/025,221   11 September 1996 (11.09.96)   US

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(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, HU, ID, IL, IS, JP, KG, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TJ, TM, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: PAPILLOMAVIRUS VACCINE FORMULATION

(57) Abstract

An immunogenic composition comprising papillomavirus virus-like particles comprised of recombinant papillomavirus L proteins mixed with recombinant papillomavirus early proteins is provided.

TITLE OF THE INVENTION
PAPILLOMAVIRUS VACCINE FORMULATION

BRIEF DESCRIPTION OF THE DRAWINGS

5   Figure 1 shows the effects of L1, L2 and E protein immunization on CRPV induced papilloma regression.

         Figure 2 shows antibody titers in rabbits immunized with VLPs and E-proteins.

         Figure 3 shows the effects of L1/L2 VLPs and E proteins
10   on papilloma development when animals were challenged with undiluted virus stock.

         Figure 4 shows the effects of L1/L2 VLPs and E proteins on papilloma development when animals were challenged with virus stock diluted 1:4.

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BACKGROUND OF THE INVENTION

         The papillomaviruses (PV) are a group of viruses that produce papillomas or warts in a variety of higher vertebrates, including humans, cattle, rabbits, horses, dogs, and nonhuman primates.
20   In addition, papillomaviruses have been linked to a variety of naturally-occurring cancers in various animal species, including humans.

         Papillomaviruses are small, nonenveloped, double-stranded DNA viruses with icosahedral symmetry that replicate solely in the nucleus of infected cells. The papillomaviruses (PVs) are remarkably
25   tissue- and species-specific (Howley, 1990). Most can replicate only in squamous epithelial cells, and there are no described instances of a papillomavirus from one species causing a productive infection in a different species (Howley, 1990). Thus, humans are the only natural hosts for human papillomaviruses (HPVs).

30   More than 75 different types of HPVs have been identified. Different HPV types are characteristically associated with specific lesions. Thus, distinct HPV types are responsible for common skin warts, plantar warts, respiratory papillomas of juvenile onset, squamous cell carcinomas arising in patients with epidermodysplasia

verruciformis (a rare dermatological disorder), and genital warts. Among the HPVs that infect the lower genital tract, HPV-6 and HPV-11 are frequently associated with subclinical infections and condylomata acuminata or benign genital warts; HPV 6 and HPV 11 are rarely seen

5   in higher-grade neoplasias and cervical carcinomas (Shah and Howley, 1990). HPV-16 and HPV-18, on the other hand, predominate in high-grade anogenital lesions and invasive cancers of the cervix, vulva, penis, and anus (Shah and Howley, 1990).

Human papillomavirus infection of the genital tract is one

10   of the most common sexually transmitted diseases. HPV infection of the genital tract may be easily recognized by the appearance of genital warts.

Several lines of evidence highlight the importance of the immune system's response, particularly cell-mediated immunity, in the

15   course of papillomavirus infections (Shah and Howley, 1990). Individuals having depressed T-cell function (e.g., AIDS patients, pregnant women, and patients undergoing immunosuppressive therapy or organ transplantation) are reported to have a higher prevalence of warts as well as an increase in the size and number of warts. In

20   addition, warts often regress completely when the immunosuppressed state is reduced or eliminated. Finally, histological sections of regressing flat skin warts show a mononuclear cell infiltrate suggestive of an immune-mediated response.

Effective prophylactic and therapeutic vaccines containing

25   the L1 and L2 proteins of bovine papillomavirus (BPV) 2 have been formulated (Jarrett et al., 1991). Cottontail rabbits have been immunized with cottontail rabbit papillomavirus (CRPV) virus-like particles (VLPs; particles that are morphologically similar to native virions but that lack viral DNA). VLPs are formed from recombinant

30   L1 protein (L1 protein is the major component of the PV viral capsid). Immunization of rabbits with CRPV VLPs (Christensen et al., 1996) or with CRPV L1 or L2 proteins (Lin et al., 1992) elicits neutralizing antibodies that protect rabbits from papilloma formation following

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challenge with native CRPVs. The neutralizing antibodies do not,
however, prevent disease progression in preexisting CRPV infection.

　　　　HPV infection in nonhuman animals does not produce
disease. This necessitates the use of animal PV models for the testing of
5　　candidate vaccines. The cottontail rabbit-CRPV model is a well-
characterized in vivo PV system; it displays a remarkable similarity to
the natural history of HPV-induced diseases (Höpfl et al., 1995). Both
CRPV and HPV infections have a high incidence of spontaneous
regression (10-40%) and of malignant progression of persistent
10　　papillomas (Höpfl et al., 1995). This similarity makes the CRPV model
particularly well suited for the development of a human anticancer
vaccine.

　　　　One objective of the instant application is the development
of an HPV VLP vaccine that is both prophylactic--that is, prevents HPV
15　　infection--and therapeutic--provides immunotherapy to patients with
preexisting HPV infection. In order to prevent HPV infection, the
vaccine must elicit neutralizing antibodies that preclude viral entry into
cells. As the CRPV model indicates, such antibodies are produced in
response to injection with CRPV VLPs. For the vaccine to be
20　　therapeutic, it must elicit a cell-mediated immune response targeted
toward the specific HPV antigens; such a response will kill HPV-
infected cells, thereby preventing disease progression. The induction of
cell-mediated immunity is accomplished via the injection of viral E
proteins, which must be taken up by the cell, processed, and presented
25　　complexed with the appropriate MHC Class I molecules by antigen-
presenting cells. Thus, the prophylactic/therapeutic HPV vaccine will
consist of VLPs in combination with the appropriate HPV E proteins.
In order to test this formulation we generated CRPV L1/L2 VLPs
expressed in baculovirus or yeast and E1, E2, E5, E6, and E7 proteins
30　　expressed in <u>Escherichia coli</u> for testing in the in vivo CRPV model.

SUMMARY OF THE INVENTION

An immunogenic composition comprising papillomavirus
virus-like particles comprised of recombinant papillomavirus L proteins
mixed with recombinant papillomavirus early proteins is provided.

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DETAILED DESCRIPTION OF THE INVENTION

An immunogenic composition comprising papillomavirus
virus-like particles comprised of recombinant papillomavirus L proteins
mixed with recombinant papillomavirus early proteins is provided.

10   Papillomavirus infections occur in a variety of animals,
including humans, sheep, dogs, cats, rabbits, monkeys, snakes and
cows. Papillomaviruses infect epithelial cells, generally inducing benign
epithelial or fibroepithelial tumors at the site of infection.

Papillomaviruses may be classified into distinct groups
15   based on the host that they infect. Human papillomaviruses (HPV) are
further classified into more than 60 types based on DNA sequence
homology (for a review, see Papillomaviruses and Human Cancer, H.
Pfister (ed.), CRC Press, Inc., 1990). Papillomavirus types appear to be
type-specific immunogens in that a neutralizing immunity to infection to
20   one type of papillomavirus does not confer immunity against another
type of papillomavirus.

In humans, different HPV types cause distinct diseases.
HPV types 1, 2, 3, 4, 7, 10 and 26-29 cause benign warts in both normal
and immunocompromised individuals. HPV types 5, 8, 9, 12, 14, 15,
25   17, 19-25, 36 and 46-50 cause flat lesions in immunocompromised
individuals. HPV types 6, 11, 34, 39, 41-44 and 51-55 cause
nonmalignant condylomata of the genital and respiratory mucosa. HPV
types 16 and 18 cause epithelial dysplasia of the genital tract and are
associated with the majority of in situ and invasive carcinomas of the
30   cervix, vagina, vulva and anal canal. HPV6 and HPV11 cause the
majority of genital warts and laryngeal papillomas.

Immunological studies in animals have shown that the
production of neutralizing antibodies to papillomavirus capsid proteins
prevents infection with the homologous virus. The development of

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effective papillomavirus vaccines has been slowed by difficulties associated with the cultivation of papillomaviruses in vitro. The development of an effective HPV vaccine has been particularly slowed by the absence of a suitable animal model.

5   Neutralization of papillomavirus by antibodies appears to be type-specific and dependent upon conformational epitopes on the surface of the virus.

Papillomaviruses are small (50-60 nm), nonenveloped, icosahedral DNA viruses that encode for up to eight early and two late

10   genes. The open reading frames (ORFs) of the virus genomes are designated E1 to E7 and L1 and L2, where "E" denotes early and "L" denotes late. L1 and L2 code for virus capsid proteins. The early (E) genes are associated with functions such as viral replication and transformation.

15   The L1 protein is the major capsid protein and has a molecular weight of 55-60 kDa. L2 protein is a minor capsid protein which has a predicted molecular weight of 55-60 kDa and an apparent molecular weight of 75-100 kDa as determined by polyacrylamide gel electrophoresis.

20   The present invention is directed to the vaccines and immunogenic compositions comprising mixtures of recombinant papillomavirus proteins having the immunity conferring properties of native papillomaviruses. The present invention is particularly directed to prophylactic and therapeutic vaccine formulations. The present

25   invention is exemplified by a cottontail rabbit papillomavirus (CRPV) model system. The exemplification does not limit the scope of the invention, which includes other types and subtypes of papillomavirus (PV), including but not limited to HPV type 11, HPV type 16 and HPV type 18 as well as HPV subtype 6a and HPV subtype 6b.

30   Pharmaceutically useful compositions comprising the proteins or VLP may be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier. Examples of such carriers and methods of formulation may be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically

acceptable composition suitable for effective administration, such compositions will contain an effective amount of the protein or VLP. Such compositions may contain proteins or VLP derived from more than one type of HPV.

5   Therapeutic or diagnostic compositions of the invention are administered to an individual in amounts sufficient to treat or diagnose PV infections. The effective amount may vary according to a variety of factors such as the individual's condition, weight, sex and age. Other factors include the mode of administration. Generally, the compositions
10   will be administered in dosages ranging from about 1 μg to about 250 μg.

The pharmaceutical compositions may be provided to the individual by a variety of routes such as subcutaneous, topical, oral, mucosal, and intramuscular.

15   The vaccines of the invention comprise recombinant proteins or VLP that contain the antigenic determinants necessary to induce the formation of neutralizing antibodies in the host. Such vaccines are also safe enough to be administered without danger of clinical infection; do not have toxic side effects; can be administered by
20   an effective route; are stable; and are compatible with vaccine carriers.

The vaccines may be administered by a variety of routes, such as orally, parenterally, subcutaneously, mucosally or intramuscularly. The dosage administered may vary with the condition, sex, weight, and age of the individual; the route of administration; and
25   the type PV of the vaccine. The vaccine may be used in dosage forms such as capsules, suspensions, elixirs, or liquid solutions. The vaccine may be formulated with an immunologically acceptable carrier.

The vaccines are administered in therapeutically effective amounts, that is, in amounts sufficient to generate a immunologically
30   protective response. The therapeutically effective amount may vary according to the type of PV. The vaccine may be administered in single or multiple doses.

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The methods of the present invention make possible the formulation of subviral vaccines for preventing or treating PV infection.

5   The recombinant proteins and VLP of the present invention may be used in the formulation of immunogenic compositions. Such compositions, when introduced into a suitable host, are capable of inducing an immunologic response in the host.

The recombinant proteins and VLP may be used to generate antibodies. The term "antibody" as used herein includes both
10   polyclonal and monoclonal antibodies, as well as fragments thereof, such as, Fv, Fab and F(ab)2 fragments that are capable of binding antigen or hapten.

The following examples are provided to further define the invention without, however, limiting the invention to the particulars of
15   these examples.

EXAMPLE 1
Subcloning and expression of CRPV E1, E2, E4, E5, E6 and E7 genes in E. coli

20   PCR primers based on the published sequence of CRPV (Yaniv, M., Danos, O. and Giri, I., "Genomic Structure of the Cottontail Rabbit (Shope) Papillomavirus", Proc. Natl. Acad. Sci. U.S.A., 82, 1580-1584 (1985)) were used to PCR amplify the full length CRPV E1, E2, E4, E5, E6 and E7 genes. To enhance expression
25   of CRPV E4 protein, the first 4 amino acid codons of CRPV E1 protein were fused to the amino terminal portion of E4 using PCR (E1^4). To co-express E6 and E7 the two genes were fused at the carboxy terminus of E6 with the amino terminus of E7 using PCR. All PCR amplified products were subcloned into the vector pQE30 (Qiagen, Inc., San
30   Diego, CA) and then transformed into E. coli.

To express these genes 1 Liter cultures of E.coli expressing desired E-proteins were grown for eight hours at 37°C in LB media and then induced overnight at 30°C using 1 mM IPTG. The cells were then collected by centrifugation for 15 min at 5000 rpm, washed with 500 ml

of PBS and the E proteins were purified using the manufacturer's directions (Qiagen, Inc.)

EXAMPLE 2

5   Subcloning and expression of CRPV L1 and L2 genes as virus like particles (VLPs)

PCR primers based on the published sequence (Yaniv, M., Danos, O. and Giri, I., "Genomic Structure of the Cottontail Rabbit (Shope) Papillomavirus", Proc. Natl. Acad. Sci. U.S.A., 82, 1580-1584

10   (1985)) of CRPV were used to PCR amplify the full length CRPV L1 gene and a CRPV L2 gene that had the first 37 codons (111 bp) deleted from the virus genome. These genes were subcloned into the 2 cassette vector pAcUW51 (PharMingen Inc., San Diego, CA) for co-expression in the baculovirus expression system or into a 2 cassette vector pGAL-

15   110 (K. J. Hofmann et al. J. Virol. 209:506-518, 1995) for co-expression in yeast.

The pAcUW51 vector containing the genes encoding CRPV L1 and L2 proteins was transfected into SF9 cells using the PharMingen Baculogold expression kit. Supernatants from this transfection were

20   used to infect large cultures (1 Liter) of Sf9 cells which grown for 5 days, the cells were collected and the L1/L2 VLPs were purified.

Purification of VLPs from Sf-9 cells:

Cells were collected by centrifugation at 3800 x g for 30

25   min and were resuspended in phosphate-buffer saline pH 7.2 (PBS) at a density of 5 x 10^7 cells/ml. 10% NP-40 was added to 2% final volume (v/v) and the cells were stirred for 20 min in cold room. The cell slurry was then centrifuged at 12,000 x g for 30 min; the nuclear pellet was then collected and resuspended in PBS as above. Nuclei were

30   sonicated for 30 sec., and then $MgCl_2$ was added (to 3 mM), Benzonase was added (to 100 units/ml) and Pefabloc was added (to 0.2 mM). The nuclei were incubated for 6 hrs at room temperature while being mixed and then centrifuged at 12,000 x g for 30 min. Supernatant was collected and CsCl was added to the supernatant to a final concentration

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of 0.48g/ml, centrifuged in a SW41 rotor at 30,000 rpm for 50 hrs and the VLP band was collected and dialyzed against PBS to remove CsCl. VLPs were further purified by anion exchange chromatography.

5   Expression of L1/L2 VLPs in yeast:

The pGAL-110 vector containing the genes encoding CRPV L1 and L2 proteins was transformed into yeast using the standard spheroplast transformation protocol. Positive clones were identified and large scale cultures were grown. 200 ml of culture media (6.7 g/L

10   Yeast Nitrogen Base without amino acids or ammonium sulfate, 0.3 g/L adenine, 0.2 g/L L-tyrosine, 0.16 g/L uracil, 7.9 g/L succinic acid, 0.1 g/L L-arginine, 0.05 g/L L-Histidine, 0.3 g/L L-isoleucine, 0.2 g/L L-lysine, 0.05 g/L L-methionine, 0.3 g/L L-phenylalanine, 0.2 g/L L-tryptophan, 4% glucose) were inoculated with L1/L2- expressing yeast

15   cultures (recombinant yeast transformed with L1/L2/pLS110 vector) and grown for 2 days at 30°C. This 200 ml culture was then used to inoculate 1 liter of induction medium (2% yeast extract, 1% soy peptone, 1.6% glucose, 4% galactose) which was grown at 30°C for 5 days. The cells were collected by centrifugation and the L1/L2 VLPs

20   were purified by the procedure described above.

EXAMPLE 3
Immunization of Rabbits with Candidate Vaccine

Rabbits were immunized with 50 ug of each CRPV L1/L2,

25   L1/L2+50 ug of each E1 and E2 or 50 ug of each E1 and E2 in 1.0 ml (0.3 ml each intramuscularly into each hind leg, 0.05ml each intradermally at 6 sites and 0.1 ml subcutaneously in the neck) of RIBI triple mix (Monophosphoryl Lipid A, Synthetic Trehalose Dicorynomycolate and Cell Wall Skeleton, RIBI ImmunoChem

30   Research, Inc. Hamilton, MT). Rabbits were infected with cottontail rabbit papillomavirus (CRPV) 4 days after the first immunization and boosted with the same amount of antigen in the same formulation on day 21 and day 70. Size of the papillomas was determined at two week intervals beginning six weeks after the virus infection.

EXAMPLE 4

We tested in cottontail rabbits the ability of vaccination with nonstructural cottontail rabbit papillomavirus (CRPV) proteins E1 and E2, alone or in combination with L1/L2 VLPs, in limiting CRPV-

5   induced papilloma development. Rabbits were immunized, using RIBI adjuvant, with L1/L2 VLPs, E1+E2 proteins, or the combination of VLPs and E-proteins, then boosted 3 weeks later with a similar dose of antigens in the same formulation. Animals were challenged 4 days after the initial antigen injection with two different concentrations of the

10   CRPV virus. Rabbits vaccinated with L1/L2 VLPs or E.coli expressed E-proteins responded with antibody titers to the respective antigens, and developed papillomas at the sites of both high and low dose virus challenge. The rates of papilloma development in these vaccinees were indistinguishable from those of the unimmunized animal controls.

15   However, the RIBI-adjuvanted vaccination of animals with L1/L2 VLPs in combination with E.coli expressed E1 and E2 proteins resulted in the retardation of papilloma development. In 5 of 9 animals the papillomas were reduced in size by greater than 80%, when compared with those of the control animals. This outcome suggests that the immunization with

20   VLPs and E-proteins induced potent immune responses to virus-infected cells. This vaccination protocol had minimal to no effect on the papilloma development on the remaining 4 immunized rabbits. We hypothesize that this result may be due to variations in their major histocompatibility complex (MHC) receptors. These studies are

25   continuing, evaluating additional adjuvants and E proteins. Studies are ongoing which investigate the utility of alternative adjuvants and E-proteins. Experiments are underway in which rabbits have been immunized with a cocktail of E1+E2+E1^4, E6^7 and E5 proteins in combination with VLPs in RIBI. Immunization of rabbits with VLPs

30   and E1 and E2 proteins formulated in 0.1% Polyphosphazine also inhibits CRPV induced wart development in 3 of 4 rabbits. However, the magnitude and duration of inhibition is less than one observed in animals immunized with the same dose in RIBI.

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WHAT IS CLAIMED IS:

1.   An immunogenic composition comprising virus-like particles containing papillomavirus L1 protein, papillomavirus L2 protein and at least one papillomavirus early protein.

2.   The composition of Claim 1 wherein the early protein is selected from papillomavirus E1, E1^4, E2, E3, E4, E5, E6, and E7 proteins.

3.   The composition of Claim 2 wherein the papillomavirus is selected from human papillomavirus and cottontail rabbit papillomavirus.

4.   The composition of Claim 1 which is a vaccine.

5.   The composition of Claim 2 which is a vaccine.

6.   The composition of Claim 3 which is a vaccine.

7.   A method of inducing an antipapillomavirus immune response in an animal comprising administration of the immunogenic composition of Claim 1 to the animal.

8.   The composition of Claim 1 which further comprises RIBI triple mix adjuvant.

9.   The composition of Claim 2 which further comprises RIBI triple mix adjuvant.

Effect of L1, L2 and E protein immunization on CRPV induced papilloma regression			
Rabbit #	Antigen	Mean Papilloma Size	Mean Papilloma Size
		LxW (mm^2)	LxWxH (mm^3)
1	RIBI	442 SD 329	4575 SD 3935
2	RIBI	326 SD 101	2229 SD 1266
3	RIBI	490 SD 11	3900 SD 962
4	RIBI+L1/L2	233 SD 122	1679 SD 1427
5	RIBI+L11/L2	547 SD 50	4626 SD 133
6	RIBI+L1/L2	697 SD 57	8042 SD 1024
7	RIBI+L1/E5–L2	427 SD 151	2723 SD 1209
8	RIBI+L1/E5–L2	744 SD 166	7438 SD 1657
9	RIBI+L1/E5–L2		
10	RIBI+E1+E2	600 SD 93	6000 SD 926
11	RIBI+E1+E2	668 SD 136	7470 SD 2213
12	RIBI+L1/L2+E1+E2	72.25 SD 64	255 SD 252
13	RIBI+L1/L2+E1+E2	600 SD 107	7700 SD 748
14	RIBI+L1/E5–L2+E1+E2	512 SD 14	3187 SD 601
15	RIBI+L1/E5–L2+E1+E2	165 SD 96	848 SD 695

FIG.1

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| Antibody titers in rabbits immunized with VLPs and E-proteins				
Rabbit #	Antigen	Anti-L1 Titers (Day 4)	Anti-E1 Titers (Day 81)	Anti-E2 Titers (Day 81)
101	RIBI	<100	<1600	<400
106	RIBI	<100	<400	<200
102	RIBI+L1/L2	<100	<200	<400
109	RIBI+L1/L2	<100	<16,00	<200
103	RIBI+L1/L2 +E1+E2	<100	12,800	25,600
105	RIBI+L1/L2 +E1+E2	<100	–	12,800
110	RIBI+L1/L2 +E1+E2	<100	3,200	12,800
113	RIBI+L1/L2 +E1+E2	<100	12,800	<25,600
114	RIBI+L1/L2 +E1+E2	200	n.d.	n.d.

FIG.2
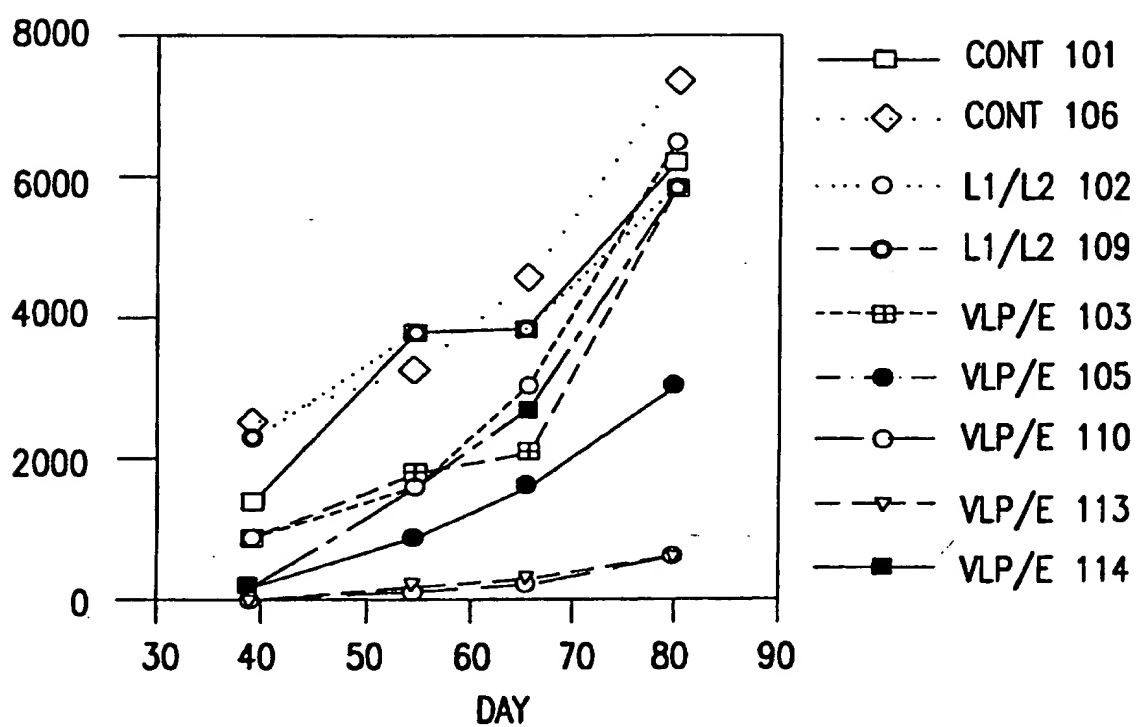
FIG.3
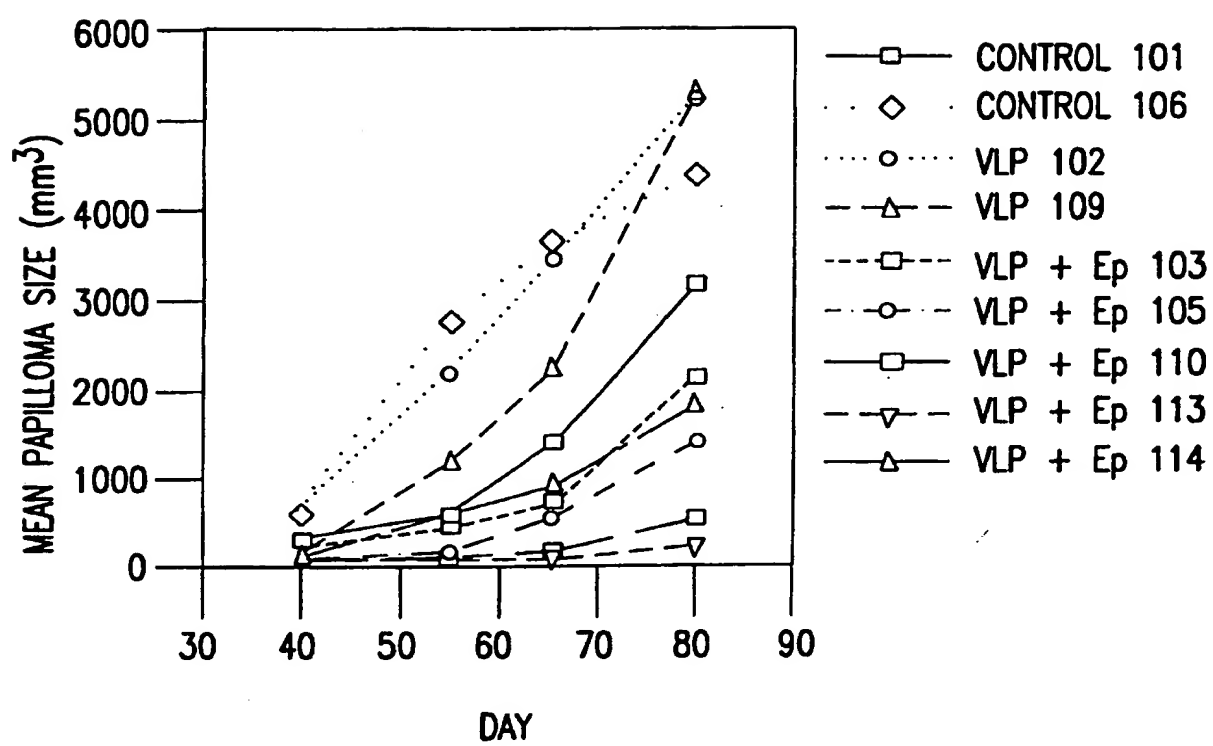
FIG.4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/15820

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/12

US CL :424/204.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/204.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	US 5,618,536 A (LOWY et al) 08 April 1997, see entire document.	1-7
X, P	MULLER et al. Chimeric Papillomavirus-like Particles. Virology. 21 July 1997, Vol. 234, pages 93-111, see entire document.	1-7
X	DE 44 35 907 A1 (GISSMANN et al) 11 April 1996, see entire document.	1-7
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Y		8-9
Y	WO 96/26277 A1 (CANTAB PHARMACEUTICALS RESEARCH LIMITED) 29 August 1996, see entire document, especially claims 4 and 13-15.	1-9

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

<table>
<tr><td>*</td><td>Special categories of cited documents:</td><td>"T"</td><td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td></tr>
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<tr><td>"P"</td><td>document published prior to the international filing date but later than the priority date claimed</td><td>"&"</td><td>document member of the same patent family</td></tr>
</table>

Date of the actual completion of the international search	Date of mailing of the international search report
12 NOVEMBER 1997	1 2 DEC 1997

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer MARY E. MOSHER, PH.D.
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document. with indication. where appropriate. of the relevant passages	Relevant to claim No.
Y	BREITBURD et al. Immunization with Viruslike Particles from Cottontail Rabbit Papillomavirus (CRPV) Can Protect Against Experimental CRPV Infection. Journal of Virology. June 1995, Vol. 69, No. 6, pages 3959-3963, see entire document.	1-9

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS. MEDLINE. DERWENT WORLD PATENTS INDEX. SEARCH TERMS: HPV, HUMAN, PAPILLOMA?. E1.
E2. E3. E4. E5. E6. E7. L1. L2. ANTIBODY, ANTIBODIES, ANTIGEN. ANTIGENS, ANTIGENIC. VACCINE,
VACCINES. RIBI TRIPLE MIX.

PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [6] : C12N 15/62, A61K 39/12	A1	(11) International Publication Number: **WO 99/18220** (43) International Publication Date: 15 April 1999 (15.04.99)

(21) International Application Number: PCT/US98/20965

(22) International Filing Date: 6 October 1998 (06.10.98)

(30) Priority Data:
08/944,368   6 October 1997 (06.10.97)   US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PAPILLOMA VIRUS CAPSOMERE VACCINE FORMULATIONS AND METHODS OF USE

(57) Abstract

Vaccine formulations comprising viral capsomeres are disclosed along with methods for their production. Therapeutic and prophylactic methods of use for the vaccine formulations are also disclosed.

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- 1 -

PAPILLOMA VIRUS CAPSOMERE VACCINE FORMULATIONS AND METHODS OF USE

FIELD OF THE INVENTION

The present invention relates to vaccine formulations
5   comprising papilloma virus proteins, either as fusion proteins, truncated proteins, or truncated fusion proteins. The invention further embraces methods for producing capsomeres of the formulations, as well as prophylactic and therapeutic methods for their use.

BACKGROUND

10   Infections with certain high-risk strains of genital papilloma viruses in humans (HPV) -- for example. HPV 16, 18, or 45 -- are believed to be the main risk factor for the formation of malignant tumors of the anogenital tract. Of the possible malignancies, cervical carcinoma is by far the most frequent: according to an estimate by the World Health
15   Organization (WHO). almost 500.000 new cases of the disease occur annually. Because of the frequency with which this pathology occurs, the connection between HPV infection and cervical carcinoma has been extensively examined. leading to numerous generalizations.

For example. precursor lesions of cervical intraepithelial
20   neoplasia (CIN) are known to be caused by papilloma virus infections [Crum, *New Eng. J. Med. 310*:880-883 (1984)]. DNA from the genomes of certain HPV types. including for example. strains 16, 18, 33, 35, and 45. have been detected in more than 95% of tumor biopsies from patients with this disorder, as well as in primary cell lines cultured from the
25   tumors. Approximately 50 to 70% of the biopsied CIN tumor cells have been found to include DNA derived only from HPV 16.

The protein products of the HPV 16 and HPV 18 early genes E6 and E7 have been detected in cervical carcinoma cell lines as well as in

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human keratinocytes transformed *in vitro* [Wettstein, *et al.*, in PAPILLOMA
VIRUSES AND HUMAN CANCER, Pfister (Ed.),CRC Press: Boca Raton, FL
1990 pp 155-179] and a significant percentage of patients with cervical
carcinoma have anti-E6 or anti-E7 antibodies.   The E6 and E7 proteins

5   have been shown to participate in induction of cellular DNA synthesis in
human cells, transformation of human keratinocytes and other cell types,
and tumor formation in transgenic mice [Arbelt, *et al.*, *J. Virol.*, *68*:4358-
4364 (1994): Auewarakul, *et al.*, *Mol. Cell. Biol.* *14*:8250-8258 (1994);
Barbosa, *et al.*, *J. Virol.* *65*:292-298 (1991); Kaur, *et al.*, *J. Gen. Virol.*

10   *70*:1261-1266 (1989): Schlegel, *et al.*, *EMBO J.*, *7*:3181-3187 (1988)].
The constitutive expression of the E6/E7 proteins appears to be necessary
to maintain the transformed condition of HPV-positive tumors.

         Despite the capacity of some HPV strains to induce
neoplastic phenotypes *in vivo* and *in vitro*. still other HPV types cause

15   benign genital warts such as condylomata acuminata and are only rarely
associated with malignant tumors [Ikenberg. In Gross, *et al.*, (eds.)
GENITAL PAPILLOMAVIRUS INFECTIONS. Springer Verlag: Berlin, pp., 87-
112].   Low risk strains of this type include, for example, HPV 6 and 11.

         Most often, genital papilloma viruses are transmitted between

20   humans during intercourse which.in many instances leads to persistent
infection in the anogenital mucous membrane.   While this observation
suggests that either the primary infection induces an inadequate immune
response or that the virus has developed the ability to avoid immune
surveillance, other observations suggest that the immune system is active

25   during primary manifestation as well as during malignant progression of
papilloma virus infections [Altmann *et al.* in VIRUSES AND CANCER,
Minson *et al.*, (eds.) Cambridge University Press, (1994) pp. 71-80].

         For example, the clinical manifestation of primary infection
by rabbit and bovine papilloma virus can be prevented by vaccination with

30   wart extracts or viral structural proteins [Altmann, *et al.*, *supra*; Campo,

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Curr. Top. In Microbiol and Immunol. 186:255-266 (1994); Yindle and
Frazer, *Curr. Top. In Microbiol. and Immunol.* 186;217-253 (1994)].
Rodents previously vaccinated with vaccinia recombinants encoding HPV
16 early proteins E6 or E7, or with synthetic E6 or E7 peptides, are

5 similarly protected from tumor formation after inoculation of HPV 16
transformed autologous cells [Altman, *et al., supra*; Campo, *et al., supra*;
Yindle and Frazer, *et al. supra*]. Regression of warts can be induced by
the transfer of lymphocytes from regressor animals following infection by
animal papilloma viruses. Finally. in immunosuppressed patients, such as,

10 for example, recipients of organ transplants or individuals infected with
HIV, the incidence of genital warts. CIN. and anogenital cancer is
elevated.

To date. no HPV vaccinations have been described which
comprise human papilloma virus late L1 protein in the form of capsomeres

15 which are suitable both for prophylactic and therapeutic purposes. Since
the L1 protein is not present in malignant genital lesions, vaccination with
L1 protein does not have any therapeutic potential for these patients.
Construction of chimeric proteins comprising amino acid residues from L1
protein and, for example E6 or E7 protein, which give rise to chimeric

20 capsomeres, combines prophylactic and therapeutic functions of a vaccine.
A method for high level production of chimeric capsomeres would
therefore be particularly desirable. in view of the possible advantages
offered by such a vaccine for prophylactic and therapeutic intervention.

Thus there exists a need in the art to provide vaccine

25 formulations which can prevent or treat HPV infection. Methods to
produce vaccine formulations which overcome problems known in the art
to be associated with recombinant HPV protein expression and purification
would manifestly be useful to treat the population of individuals already
infected with HPV as well as useful to immunize the population of

30 individuals susceptible to HPV infection.

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SUMMARY OF THE INVENTION

The present invention provides therapeutic and prophylactic vaccine formulations comprising chimeric human papilloma capsomeres. The invention also provides therapeutic methods for treating patients

5   infected with an HPV as well as prophylactic methods for preventing HPV infection in a susceptible individual. Methods for production and purification of capsomeres and proteins of the invention are also contemplated.

In one aspect of the invention, prophylactic vaccinations for

10   prevention of HPV infection are considered which incorporate the structural proteins L1 and L2 of the papilloma virus. Development of a vaccine of this type faces significant obstacles because papilloma viruses cannot be propagated to adequate titers in cell cultures or other experimental systems to provide the viral proteins in sufficient quantity for economical vaccine

15   production. Moreover. recombinant methodologies to express the proteins are not always straightforward and often results in low protein yield. Recently. virus-like particles (VLPs). similar in make up to viral capsid structures. have been described which are formed in Sf-9 insect cells upon expression of the viral proteins L1 and L2 (or L1 on its own) using

20   recombinant vaccinia or baculovirus. Purification of the VLPs can be achieved very simply by means of centrifugation in CsCl or sucrose gradients [Kimbauer. *et al.. Proc. Natl. Acad. Sci. (USA)*, *99*:12180-12814 (1992): Kimbaurer, *et al., J. Virol.* *67*:6929-6936 (1994); Proso, *et al., J. Virol.* *6714*:1936-1944 (1992): Sasagawa. *et al., Virology 2016*:126-195

25   (1995): Volpers, *et al., J. Virol. 69*:3258-3264 (1995); Zhou, *et al., J. Gen. Virol. 74*:762-769 (1993): Zhou, *et al., Virology 185*:251-257 (1991)]. WO 93/02184 describes a method in which papilloma virus-like particles (VLPs) are used for diagnostic applications or as a vaccine against infections caused by the papilloma virus. WO 94/00152 describes

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recombinant production of L1 protein which mimics the conformational
neutralizing epitope on human and animal papilloma virions.

In another aspect of the invention, therapeutic vaccinations
are provided to relieve complications of, for example, cervical carcinoma
5   or precursor lesions resulting from papilloma virus infection, and thus
represent an alternative to prophylactic intervention. Vaccinations of this
type may comprise early papilloma virus proteins, principally E6 or E7,
which are expressed in the persistently infected cells. It is assumed that,
following administration of a vaccination of this type, cytotoxic T-cells
10   might be activated against persistently infected cells in genital lesions. The
target population for therapeutic intervention is patients with HPV-
associated pre-malignant or malignant genital lesions. PCT patent
application WO 93/20844 discloses that the early protein E7 and antigenic
fragments thereof of the papilloma virus from HPV or BPV is
15   therapeutically effective in the regression, but not in the prevention, of
papilloma virus tumors in mammals. While early HPV proteins have been
produced by recombinant expression in *E. coli* or suitable eukaryotic cell
types, purification of the recombinant proteins has proven difficult due to
inherent low solubility and complex purification procedures which generally
20   require a combination of steps, including ion exchange chromatography, gel
filtration and affinity chromatography.

According to the present invention, vaccine formulations
comprising papilloma virus capsomeres are provided which comprise
either: (i) a first protein that is an intact viral protein expressed as a fusion
25   protein comprised in part of amino acid residues from a second protein; (ii)
a truncated viral protein; (iii) a truncated viral protein expressed as a fusion
protein comprised in part of amino acid residues from a second protein, or
(iv) some combination of the three types of proteins. According to the
invention, vaccine formulations are provided comprising capsomeres of
30   bovine papilloma virus (BPV) and human papilloma virus. Preferred

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bovine virus capsomeres comprise protein from bovine papilloma virus type I. Preferred human virus capsomeres comprise proteins from any one of human papilloma virus strains HPV6, HPV11, HPV16, HPV18, HPV33, HPV35, and HPV45. The most preferred vaccine formulations comprise

5   capsomeres comprising proteins from HPV16.

In one aspect, capsomere vaccine formulations of the invention comprise a first intact viral protein expressed as a fusion protein with additional amino acid residues from a second protein. Preferred intact viral proteins are the structural papilloma viral proteins L1 and L2.

10   Capsomeres comprised of intact viral protein fusions may be produced using the L1 and L2 proteins together or the L1 protein alone. Preferred capsomeres are made up entirely of L1 fusion proteins, the amino acid sequence of which is set out in SEQ ID NO: 2 and encoded by the polynucleotide sequence of SEQ ID NO: 1. Amino acids of the second

15   protein can be derived from numerous sources (including amino acid residues from the first protein) as long as the addition of the second protein amino acid residues to the first protein permits formation of capsomeres. Preferably, addition of the second protein amino acid residues inhibits the ability of the intact viral protein to form virus-like particle structures; most

20   preferably, the second protein amino acid residues promote capsomere formation. In one embodiment of the invention, the second protein may be any human tumor antigen, viral antigen, or bacterial antigen which is important in stimulating an immune response in neoplastic or infectious disease states. In a preferred embodiment, the second protein is also a

25   papilloma virus protein. It also preferred that the second protein be the expression product of papilloma virus early gene. It is also preferred, however, that the second protein be selected from group of E1, E2, E3, E4, E5, E6, and E7 — early gene products encoded in the genome of papilloma virus strains HVP6, HPV11, HPV18, HPV33, HPV35, or HPV

30   45. It is most preferred that the second protein be encoded by the HPV16

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E7 gene, the open reading frame of which is set out in SEQ ID NO: 3.
Capsomeres assembled from fusion protein subunits are referred to herein
as chimeric capsomeres. In one embodiment, the vaccine formulation of
the invention is comprised of chimeric capsomeres wherein L1 protein

5   amino acid residues make up approximately 50 to 99% of the total fusion
protein amino acid residues. In another embodiment, L1 amino acid
residues make up approximately 60 to 90% of the total fusion protein
amino acid residues; in a particularly preferred embodiment, L1 amino
acids comprise approximately 80% of the fusion protein amino acid

10   residues.

In another aspect of the invention, capsomere vaccine
formulations are provided that are comprised of truncated viral proteins
having a deletion of one or more amino acid residues necessary for
formation of a virus-like particle. It is preferred that the amino acid

15   deletion not inhibit formation of capsomeres by the truncated protein, and it
is most preferred that the deletion favor capsomere formation. Preferred
vaccine formulations of this type include capsomeres comprised of
truncated L1 with or without L2 viral proteins. Particularly preferred
capsomeres are comprised of truncated L1 proteins. Truncated proteins

20   contemplated by the invention include those having one or more amino acid
residues deleted from the carboxy terminus of the protein, or one or more
amino acid residues deleted from the amino terminus of the protein, or one
or more amino acid residues deleted from an internal region (i.e., not from
either terminus) of the protein.   Preferred capsomere vaccine formulations

25   are comprised of proteins truncated at the carboxy terminus. In
formulations including L1 protein derived from HPV16, it is preferred that
from 1 to 34 carboxy terminal amino acid residues are deleted. Relatively
shorter deletions are also contemplated which offer the advantage of minor
modification of the antigenic properties of the L1 proteins and the

30   capsomeres formed thereof. It is most preferred, however, that 34 amino

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acid residues be deleted from the L1 sequence, corresponding to amino acids 472 to 505 in HPV16 set out in SEQ ID NO: 2, and encoded by the polynucleotide sequence corresponding to nucleotides 1414 to 1516 in the human HPV16 L1 coding sequence set out in SEQ ID NO: 1.

5   When a capsomere vaccine formulation is made up of proteins bearing an internal deletion, it is preferred that the deleted amino acid sequence comprise the nuclear localization region of the protein. In the L1 protein of HPV 16, the nuclear localization signal is found from about amino acid residue 499 to about amino acid residue 505. Following

10   expression of L1 proteins wherein the NLS has been deleted, assembly of capsomere structures occurs in the cytoplasm of the host cell. Consequently, purification of the capsomeres is possible from the cytoplasm instead of from the nucleus where intact L1 proteins assemble into capsomeres. Capsomeres which result from assembly of truncated

15   proteins wherein additional amino acid sequences do not replace the deleted protein sequences are necessarily not chimeric in nature.

In still another aspect of the invention, capsomere vaccine formulations are provided comprising truncated viral protein expressed as a fusion protein adjacent amino acid residues from a second protein.

20   Preferred truncated viral proteins of the invention are the structural papilloma viral proteins L1 and L2. Capsomeres comprised of truncated viral protein fusions may be produced using L1 and L2 protein components together or L1 protein alone. Preferred capsomeres are those comprised of L1 protein amino acid residues. Truncated viral protein components of the

25   fusion proteins include those having one or more amino acid residues deleted from the carboxy terminus of the protein, or one or more amino acid residues deleted from the amino terminus of the protein, or one or more amino acid residues deleted from an internal region (i.e., not from either terminus) of the protein. Preferred capsomere vaccine formulations

30   are comprised of proteins truncated at the carboxy terminus. In those

- 9 -

formulations including L1 protein derived from HPV16, it is preferred that from 1 to 34 carboxy terminal amino acid residues are deleted. Relatively shorter deletions are also contemplated that offer the advantage of minor modification of the antigenic properties of the L1 protein component of the

5   fusion protein and the capsomeres formed thereof. It is most preferred, however, that 34 amino acid residues be deleted from the L1 sequence, corresponding to amino acids 472 to 505 in HPV16 set out in SEQ ID NO: 2, and encoded by the polynucleotide sequence corresponding to nucleotides 1414 to 1516 in the human HPV16 L1 coding sequence set out

10   in SEQ ID NO: 1. When the vaccine formulation is comprised of capsomeres made up of proteins bearing an internal deletion, it is preferred that the deleted amino acid sequence comprise the nuclear localization region, or sequence, of the protein.

Amino acids of the second protein can be derived from

15   numerous sources as long as the addition of the second protein amino acid residues to the first protein permits formation of capsomeres. Preferably, addition of the second protein amino acid residues promotes or favors capsomere formation. Amino acid residues of the second protein can be derived from numerous sources, including amino acid residues from the

20   first protein. In a preferred embodiment, the second protein is also a papilloma virus protein. It also preferred that the second protein be the expression product of papilloma virus early gene. It is most preferred, however, that the second protein be selected from group of early gene products encoding by papilloma virus E1, E2, E3, E4, E5, E6, and E7

25   genes. In one embodiment, the vaccine formulation of the invention is comprised of chimeric capsomeres wherein L1 protein amino acid residues make up approximately 50 to 99% of the total fusion protein amino acid residues. In another embodiment, L1 amino acid residues make up approximately 60 to 90% of the total fusion protein amino acid residues; in

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a particularly preferred embodiment, L1 amino acids comprise
approximately 80% of the fusion protein amino acid residues.

In a preferred embodiment of the invention, proteins of the
vaccine formulations are produced by recombinant methodologies, but in
5   formulations comprising intact viral protein, the proteins may be isolated
from natural sources. Intact proteins isolated from natural sources may be
modified *in vitro* to include additional amino acid residues to provide a
fusion protein of the invention using covalent modification techniques well
known and routinely practiced in the art. Similarly, in formulations
10   comprising truncated viral proteins. the proteins may be isolated from
natural sources as intact proteins and hydrolyzed *in vitro* using chemical
hydrolysis or enzymatic digestion with any of a number of site-specific or
general proteases. the truncated protein subsequently modified to include
additional amino acid resides as described above to provide a truncated
15   fusion protein of the invention.

In producing capsomeres. recombinant molecular biology
techniques can be utilized to produce DNA encoding either the desired
intact protein. the truncated protein. or the truncated fusion protein.
Recombinant methodologies required to produce a DNA encoding a desired
20   protein are well known and routinely practiced in the art. Laboratory
manuals. for example Sambrook. *et al.*. (eds.), MOLECULAR CLONING: A
LABORATORY MANUAL. Cold Spring Harbor Press: Cold Spring Harbor,
NY (1989) and Ausebel *et al.*. (eds.). PROTOCOLS IN MOLECULAR
BIOLOGY. John Wiley & Sons. Inc. (1994-1997), describe in detail
25   techniques necessary to carry out the required DNA manipulations. For
large-scale production of chimeric capsomeres, protein expression can be
carried out using either viral or eukaryotic vectors. Preferable vectors
include any of the well known prokaryotic expression vectors, recombinant
baculoviruses, COS cell specific vectors, vaccinia recombinants, or yeast-
30   specific expression constructs. When recombinant proteins are used to

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provide capsomeres of the invention, the proteins may first be isolated from the host cell of its expression and thereafter incubated under conditions which permit self-assembly to provide capsomeres. Alternatively, the proteins may be expressed under conditions wherein capsomeres are formed

5   in the host cell.

The invention also contemplates processes for producing capsomeres of the vaccine formulations. In one method, L1 proteins are expressed from DNA encoding six additional histidines at the carboxy terminus of the L1 protein coding sequence. L1 proteins expressed with

10   additional histidines (His L1 proteins) are most preferably expressed in *E. coli* and the His L1 proteins can be purified using nickel affinity chromatography. His L1 proteins in cell lysate are suspended in a denaturation buffer, for example. 6 M guanidine hydrochloride or a buffer of equivalent denaturing capacity. and then subjected to nickel

15   chromatography. Protein eluted from the nickel chromatography step is renatured. for example in 150 mM NaCl. 1 mM CaCl$_2$. 0.01% Triton-X 100, 10 mM HEPES (N-2-hydroxyethyl piperazine-N'-2 ethane sulfonic acid). pH 7.4. According to a preferred method of the invention, assembly of capsomeres takes place after dialysis of the purified proteins, preferably

20   after dialysis against 150 mM NaCl. 25 mM Ca^{2+}. 10% DMSO (dimethyl sulfoxide). 0.1% Triton-X 100. 10 mM Tris [tris-(hydroxymethyl) amino-methane] acetic acid with a pH value of 5.0.

Formation of capsomeres can be monitored by electron microscopy, and, in instances wherein capsomeres are comprised of fusion

25   proteins, the presence of various protein components in the assembled capsomere can be confirmed by Western blot analysis using specific antisera.

According to the present invention, methods are provided for therapeutic treatment of individuals infected with HPV comprising the step

30   of administering to a patient in need thereof an amount of a vaccine

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formulation of the invention effective to reduce the level of HPV infection. The invention also provide methods for prophylactic treatment of individuals susceptible to HPV infection comprising the step of administering to an individual susceptible to HPV infection an amount of a

5   vaccine formulation of the invention effective to prevent HPV infection. While infected individuals can be easily identified using standard diagnostic techniques, susceptible individuals may be identified, for example, as those engaged in sexual relations with an infected individual. However, due to the high frequency of HPV infection, all sexually active persons are

10 susceptible to papilloma virus infection.

         Administration of a vaccine formulation can include one or more additional components such as pharmaceutically acceptable carriers, diluents. adjuvants, and/or buffers. Vaccines may be administered at a single time or at multiple times. Vaccine formulation of the invention may

15 be delivered by various routes including. for example, oral, intravenous, intramuscular. nasal, rectal. transdermal. vaginal, subcutaneous, and intraperitoneal administration.

         Vaccine formulations of the invention offer numerous advantages when compared to conventional vaccine preparations. As part

20 of a therapeutic vaccination. capsomeres can promote elimination of persistently infected cells in. for example. patients with CIN or cervical carcinoma. Additionally. therapeutic vaccinations of this type can also serve a prophylactic purpose in protecting patients with CIN lesions from re-infection. As an additional advantage. capsomeres can escape

25 neutralization by pre-existing anticapsid antibodies and thereby posses longer circulating half-life as compared to chimeric virus-like particles.

         Vaccine formulations comprising chimeric capsomeres can provide the additional advantage of increased antigenicity of both protein components of the fusion protein from which the capsomere is formed.

30 For example, in a VLP, protein components of the underlying capsomere

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may be buried in the overall structure as a result of internalized positioning within the VLP itself. Similarly, epitopes of the protein components may be sterically obstructed as a result of capsomere-to-capsomere contact, and therefore unaccessible for eliciting an immune response. Preliminary

5   results using L1/E7 fusion proteins to produce VLPs support this position in that no antibody response was detected against the E7 component. This observation is consistent with previous results which indicate that the carboxy terminal region of L1 forms inter-pentameric arm structures that allow assembly of capsomeres into capsids [Garcia, *et al., J. Virol. 71*:

10   2988-2995 (1997)]. Presumably in a chimeric capsomere structure, both protein components of the fusion protein substructure are accessible to evoke an immune response. Capsomere vaccines would therefore offer the additional advantage of increased antigenicity against any protein component, including, for example, neutralizing epitopes from other virus

15   proteins, expressed as a fusion with L1 amino acid sequences.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is illustrated by the following examples. Example 1 describes construction of expression vectors to produce fusion, or chimeric, viral proteins. Example 2 relates to

20   generation of recombinant baculoviruses for expression of viral proteins. Example 3 addresses purification of capsomeres. Example 4 describes an immunization protocol for production of antisera and monoclonal antibodies. Example 5 provides a peptide ELISA to quantitate capsomere formation. Example 6 describes an antigen capture ELISA to quantitate

25   capsomere formation. Example 7 provides a hemagglutinin assay to assay for the induction of neutralizing antibodies.

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Example 1
Construction of Chimeric L1 Genes

DNA encoding the HPV 16 L1 open reading frame was excised
from plasmid 16-114/k-L1/L2-pSynxtVI⁻ [Kirnbauer *et al.*, *J. Virol.*

5   *67*:6929-6936 (1994)] using *Bgl*II and the resulting fragment subcloned into
pUC19 (New England Biolabs, Beverly, MA) previously linearized at the
unique *Bam*HI restriction site. Two basic expression constructs were first
generated to permit subsequent insertion of DNA to allow fusion protein
expression. One construct encoded HPV 16 L1Δ310 having a nine amino

10   acid deletion: the deleted region was known to show low level homology
with all other papilloma virus L1 proteins. The second construct, HPV 16
L1 ΔC, encoded a protein having a 34 amino acid deletion of the carboxy
terminal L1 residues. Other constructs include an *Eco*RV restriction site at
the position of the deletion for facilitated insertion of DNA encoding other

15   protein sequences. Addition of the *Eco*RV site encodes two non-L1 protein
amino acids. aspartate and isoleucine.

A. Generation of an HPV 16 L1Δ310 expression construct

Two primers (SEQ ID NOs: 5 and 6) were designed to
amplify the pUC19 vector and the complete HPV 16 L1 coding sequence,

20   except nucleotides 916 through 942 in SEQ ID NO: 1. Primers were
synthesized to also introduce a unique *Eco*RV restriction site (underlined in
SEQ ID NOs: 5 and 6) at the termini of the amplification product.

CCCC<u>GATATC</u>GCCTTTAATGTATAAATCGTCTGG
SEQ ID NO: 5

25   CCCC<u>GATATC</u>TCAAATTATTTTCCTACACCTAGTG
SEQ ID NO: 6

The resulting PCR product was digested with *Eco*RV to provide
complementary ends and the digestion product circularized by ligation.

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Ligated DNA was transformed into *E. coli* using standard techniques and plasmids from resulting colonies were screened for the presence of an *Eco*RV restriction site. One clone designated HPV 16 L1 ∆310 was identified as having the appropriate twenty-seven nucleotide deletion and

5   this construct was used to insert DNA fragments encoding other HPV 16 proteins at the *Eco*RV site as discussed below.

B. Generation of an HPV 16 L1 ∆C expression constructs

Two primers (SEQ ID NOs: 7 and 8) were designed complementary to the HPV 16 L1 open reading frame such that the primers

10   abutted each other to permit amplification in reverse directions on the template DNA comprising HPV 16 L1-encoding sequences in pUC19 described above.

AAA<u>GATATC</u>TTGTAGTAAAAATTTGCGTCCTAAAGGAAAC
SEQ ID NO: 7

15   AAA<u>GATATC</u>TAATCTACCTCTACAACTGCTAAACGCAAAAAACG
SEQ ID NO: 8

Each primer introduced an *Eco*RV restriction site at the terminus of the amplification product. In the downstream primer (SEQ ID NO: 8), the *Eco*RV site was followed by a TAA translational stop codon positioned

20   such that the amplification product, upon ligation of the *Eco*RV ends to circularize, would include deletion of the 34 carboxy terminal L1 amino acids. PCR was performed to amplify the partial L1 open reading frame and the complete vector. The amplification product was cleaved with *Eco*RV, circularized with T4 DNA ligase, and transformed into *E. coli*

25   DH5 α cells. Plasmids from viable clones were analyzed for the presence of an *Eco*RV site which would linearize the plasmid. One positive

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construct designated pUCHPV16L1ΔC was identified and used to insert DNA from other HPV 16 proteins utilizing the *Eco*RV site.

C. Insertion of DNA fragments into HPV 16 L1 Δ310 and HPV16L1ΔC

DNA fragments of HPV 16 E7 encoding amino acids 1-50,

5   1-60. 1-98. 25-75, 40-98, 50-98 in SEQ ID NO: 4 were amplified using primers that introduced terminal 5' *Eco*RV restriction sites in order to facilitate insertion of the fragment into either HPV 16 L1 Δ310 and HPV16L1ΔC modified sequence. In the various amplification reactions, primer E7.1 (SEQ ID NO: 9) was used in combination with primer E7.2

10   (SEQ ID NO: 10) to generate a DNA fragment encoding E7 amino acids 1-50: with primer E7.3 (SEQ ID NO: 11) generate a DNA fragment encoding E7 amino acids 1-60: or with primer E7.4 (SEQ ID NO: 12) generate a DNA fragment encoding E7 amino acids 1-98. In other amplification reactions. primer pairs E7.5 (SEQ ID NO: 13) and E7.6

15   (SEQ ID NO: 14) were used to amplify a DNA fragment encoding E7 amino acids 25-75: E7.7 (SEQ ID NO: 15) and E7.4 (SEQ ID NO: 12) were used to amplify a DNA fragment encoding E7 amino acids 40-98; and E7.8 (SEQ ID NO: 16) and E7.4 (SEQ ID NO: 12) were used to amplify a DNA fragment encoding E7 amino acids 50-98.

20   Primer E7.1   SEQ ID NO: 9
         AAAAGATATCATGCATGGAGATACACCTACATTGC

     Primer E7.2   SEQ ID NO: 10
         TTTTGATATCGGCTCTGTCCGGTTCTGCTTGTCC

     Primer E7.3   SEQ ID NO: 11
25   TTTTGATATCCTTGCAACAAAAGGTTACAATATTGTAATGGGCC

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Primer E7.4   SEQ ID NO: 12
    AAAA<u>GATATC</u>TGGTTTCTGAGAACAGATGGGGCAC

Primer E7.5   SEQ ID NO: 13
    TTTT<u>GATATC</u>GATTATGAGCAATTAAATGACAGCTCAG

5   Primer E7.6   SEQ ID NO: 14
    TTTT<u>GATATC</u>GTCTACGTGTGTGCTTTGTACGCAC

Primer E7.7   SEQ ID NO: 15
    TTTATC<u>GATATC</u>GGTCCAGCTGGACAAGCAGAACCGGAC

Primer E7.8   SEQ ID NO: 16
10   TTTT<u>GATATC</u>GATGCCCATTACAATATTGTAACCTTTTG

        Similarly, nucleotides from DNA encoding the influenza
matrix protein (SEQ ID NO: 17) was amplified using the primer pair set
out in SEQ ID NOs: 19 and 20. Both primers introduced an *EcoRV*
restriction site in the amplification product.

15   TTTT<u>GATATC</u>GATATGGAATGGCTAAAGACAAGACCAATC
                                      SEQ ID NO: 19

    TTTT<u>GATATC</u>GTTGTTTGGATCCCCATTCCCATTG
                                        SEQ ID NO: 20

        PCR products from each amplification reaction were cleaved
20   with *EcoRV* and inserted into the *EcoRV* site of either the HPV 16 L1
Δ310 and HPV16L1ΔC sequences previously linearized with the same
enzyme. In order to determine the orientation of inserts in plasmids
encoding E7-amino acids 25-75 and 50-98 and plasmid including influenza
matrix protein, *ClaI* digestion was employed, taking advantage of a

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restriction site overlapping the newly created *Eco*RV restriction site (GATATCGAT) and included in the upstream primer. For the three expression constructs including the initiating methionine of HPV16 E7, insert orientation was determined utilizing a *Nsi*I restriction site within the

5   E7 coding region.

Once expression constructs having appropriate inserts were identified, the protein coding region for both L1 and inserted amino acids was excised as a unit using restriction enzymes *Xba*I and *Sma*I and the isolated DNA ligated into plasmid pVL1393 (Invitrogen) to generate

10   recombinant baculoviruses.

D. Elimination of *Eco*RV Restriction Sites in Expression Constructs

The HPV 16 L1 ΔC sequence includes DNA from the *Eco*RV site that results in translation of amino acids not normally found in wild-type L1 polypeptides. Thus. a series of expression constructions was

15   designed in which the artificial *Eco*Rv site was eliminated. The L1 sequence for this series of expression constructs was designated HPV 16L1ΔC*.

To generate an expression construct containing the HPV 16L1ΔC* sequence. two PCR reactions were performed to amplify two

20   overlapping fragments from the pUC-HPV16 L1ΔC encoding E7 amino acids 1-50. The resulting DNA fragments overlapped at the position of the L1/E7 boundary but did not contain the two *Eco*RV restriction sites. Fragment 1 was generated using primers P1 (SEQ ID NO: 21) and P2 (SEQ ID NO: 22) and fragment 2 using primers P3 (SEQ ID NO: 23) and

25   P4 (SEQ ID NO: 24).

Primer P1   SEQ ID NO: 21
                    GTTATGACATACATACATTCTATG

Primer P2   SEQ ID NO: 22

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CCATGCATTCCTGCTTGTAGTAAAAATTTGCGTCC

Primer P3   SEQ ID NO: 23
                CTACAAGCAGGAATGCATGGAGATACACC

Primer P4   SEQ ID NO: 24
        CATCTGAAGCTTAGTAATGGGCTCTGTCCGGTTCTG

Following the first two amplification reactions, the two
purified products were used as templates in another PCR reaction using
primers P1 and P4 only. The resulting amplification product was digested
with enzymes *EcoNI* and *Hind*III inserted into the HPV 16L1ΔC
expression construct described above following digestion with the same
enzymes. The resulting expression construct differed from the original
HPV16L1ΔC construct with DNA encoding L1 and E7 amino acids 1-50 by
loss of the two internal *EcoRV* restriction sites. The first *EcoRV* site was
replaced by DNA encoding native L1 alanine and glycine amino acids in
this position and the second was replaced by a translational stop signal. In
addition. the expression construct. designated HPV 16 L1ΔC* E7 1-52,
contained the first 52 amino acids of HPV 16 E7 as a result of using
primer P4 which also encodes E7 amino acids residues histidine at position
51 and tyrosine at position 52. HPV 16 L1ΔC* E7 1-52 was then used to
generate additional HPV 16 L1ΔC expression constructs further including
DNA encoding E7 amino acids 1-55 using primer P1 (SEQ ID NO: 21) in
combination with primer P5 (SEQ ID NO: 25). E7 amino acids 1-60 with
primer pair P1 and P6 (SEQ ID NO: 26). and E7 amino acids 1-65 with
primer pair P1 and P7 (SEQ ID NO: 27). The additional animo acid-
encoding DNA sequences in the amplification products arose from design
of the primers to include additional nucleotides for the desired amino acids.

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Primer P5   SEQ ID NO: 25
    CATCTGAAGCTTAACAATATTGTAATGGGCTCTGTCCG

Primer P6   SEQ ID NO: 26
    CATCTGAAGCTTACTTGCAACAAAAGGTTA-
                    CAATATTGTAATGGGCTCTGTCCG

Primer P7   SEQ ID NO: 27
    CATCTGAAGCTTAAAGCGTAGAGTCACACTTGCAAC-
                    AAAAGGTTACAATATTGTAATGGGCTCTGTCCG

Similarly, HPV 16 L1ΔC* E7 1-70 was generated using template DNA
encoding HPV 16 L1ΔC* E7 1-66 and the primer pair P1 and P8 (SEQ ID
NO: 28).

Primer P8   SEQ ID NO: 28
        CATCTGAAGCTTATTGTACGCACAAC-
                    CGAAGCGTAGAGTCACACTTG

Following each PCR reaction. the amplification products were digested
with EcoNI and HindIII and inserted into HPV16L1ΔC previously digested
with the same enzymes. Sequences of each constructs were determined
using an Applied Biosystems Prism 377 sequencing instrument with
fluorescent chain terminating dideoxynucleotides [Prober et al., Science
238:336-341 (1987)].

Example 2
Generation of Recombinant Baculoviruses

Spodoptera frugiperda (Sf9) cells were grown in suspension or
monolayer cultures at 27° in TNMFH medium (Sigma) supplemented with
10% fetal calf serum and 2 mM glutamine. For HPV 16 L1-based
recombinant baculovirus construction, Sf9 cells were transfected with 10 μg
of transfer plasmid together with 2 μg of linearized Baculo-Gold DNA

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(PharMingen, San Diego, CA). Recombinant viruses were purified by according to manufacturer's suggested protocol.

To test for expression of HPV 16 L1 protein, 10^5 Sf9 cells were infected with baculovirus recombinant at a multiplicity of infection
5   (m.o.i) of 5 to 10. After incubation for three to four days at 28°C, media was removed and cells were washed with PBS. The cells were lysed in SDS sample buffer and analyzed by SDS-PAGE and Western blotting using anti-HPV16 L1 and anti-HPV16 E7 antibodies.

In order to determine which of the chimeric L1 protein
10   expression constructs would preferentially produce capsomeres, extracts from transfected cells were subjected to gradient centrifugation. Fractions obtained from the gradient were analyzed for L1 protein content by Western blotting and for VLP formation by electron microscopy. The , results are shown in Table 1.

15   The intact HPV L1 protein. as well as the expression products HPV 16 L1Δ310 and HPV 16 L1ΔC. each were shown to produce capsomeres and virus-like particles in equal proportions. When E7 coding sequences were inserted into the HPV 16 L1Δ310 vector, only fusion proteins including E7 amino acids 1 to 50 produced gave rise to detectable
20   capsomere formation.

When E7 encoding DNA was inserted into the HPV 16 L1ΔC vector, all fusion proteins were found to produce capsomeres; chimeric proteins including E7 amino acid residues 40-98 produced the highest level of exclusively capsomere structures. Chimeric proteins
25   including E7 amino acids 1-98 and 25-75 both produced predominantly capsomeres, even thorough virus-like particle formation was also observed. The chimeric protein including E7 amino acids 1-60 resulted in nearly equal levels of capsomere and virus-like particle production.

When E7 sequences were inserted into the HPV 16 L1Δ*C
30   vector, all fusion proteins were shown to produce capsomeres. Insertion of

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DNA encoding E7 residues 1-52, 1-55, and 1-60 produced the highest level
of capsomeres, but equal levels of virus-like particle production were
observed. While insertion of DNA encoding E7 DNA for residues 1-65, 1-
70, 25-75, 40-98, and 1-98 resulted in comparatively lower levels or
5 undetectable levels of capsid, capsomeres were produced in high quantities.

TABLE 1

Capsomeree and Capsid Forming Capacity of
Chimeric HPV L1 Proteins

L1 Expression Construct	Insert	Capsomere Yield	Capsid Yield
HVP 16 L1	None	+ + + +	+ + + +
HPV 16 L1Δ310	None	+ + +	+ +
HPV 16 L1ΔC	None	+ + + +	+ + + +
HPV 16 L1Δ310	E7 1-98	-	-
HPV 16 L1Δ310	E7 1-50	+ +	-
HPV 16 L1Δ310	E7 25-75	-	-
HPV 16 L1Δ310	E7 50-98	-	_/
HPV 16 L1ΔC	E7 1-98	+ + +	+
HPV 16 L1ΔC	E7 25-75	+ + +	+
HPV 16 L1ΔC	E7 50-98	+	+
HPV 16 L1ΔC	E7 1-60	+ + + + +	+ + + + +
HPV 16 L1ΔC	E7 40-98	+ + + +	-
HPV 16 L1ΔC	Influenza	+ + +	+
HPV 16 L1Δ*C	E7 1-52	+ + + + +	+ + + + +
HPV 16 L1Δ*C	E7 1-55	+ + + + +	+ + + + +
HPV 16 L1Δ*C	E7 1-60	+ + +	+ + + +
HPV 16 L1Δ*C	E7 1-65	+ +	-
HPV 16L1Δ*C	E7 1-70	+ +	-

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Example 3
Purification of Capsomeres

Trichopulsia ni (TN) High Five cells were grown to a density of approximately 2 x 10^6 cells/ml in Ex-Cell 405 serum-free medium (JRH

5   Biosciences). Approximately 2 x 10^8 cells were pelleted by centrifugation at 1000 x g for 15 minutes. resuspended in 20 ml of medium, and infected with recombinant baculoviruses at m.o.i of 2 to 5 for 1 hour at room temperature. After addition of 200 ml medium. cells were plated and incubated for 3 to 4 days at 27°C. Following incubation. cells were harvested, pelleted, and resuspended in

10   10 ml of extraction buffer.

The following steps were performed at 4°C. Cells were sonicated for 45 seconds at 60 watts and the resulting cell lysate was centrifuged at 10.000 rpm in a Sorval SS34 rotor. The supernatant was removed and retained while the resulting pellet was resuspended in 6 ml of extraction buffer. sonicated for an

15   additional 3 seconds at 60 watts. and centrifuged again. The two supernatants were combined, layered onto a two-step gradient containing 14 ml of 40% sucrose on top of 8 ml of CsCl solution (4.6 g CsCl per 8 ml in extraction buffer). and centrifuged in a Sorval AH629 swinging bucket rotor for 2 hours at 27.000 rpm at 10°C. The interface region between the CsCl and the sucrose along with the CsCl

20   complete layer were collected into 13.4 ml Quickseal tubes (Beckman) and extraction buffer added to adjust the volume 13.4 ml. Samples were centrifuged overnight at 50.000 rpm at 20°C in a Beckman 70 TI rotor. Gradients were fractionated (1 ml per fraction) by puncturing tubes on top and bottom with a 21-gauge needle. Fractions were collected from each tube and 2.5 μl of each fraction

25   were analyzed by a 10% SDS-polyacrylamide gel and Western blotting using an anti-HPV16 L1 antibody.

Virus-like particles and capsomeres were separated from the fractions identified above by sedimentation on 10 to 50% sucrose gradients. Peak fractions from CsCl gradients were pooled and dialyzed for 2 hours against 5 mM HEPES

30   (pH 7.5). Half of the dialysate was used to produce capsomeres by disassembly of intact VLPs overnight by adding EDTA (final concentration 50 mM), EGTA (50

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mM), DTT (30 mM). NaCl (100 mM), and Tris/HCl, pH 8.0, (10 mM). As
control, NaCl and Tris/HCl only were added to the other half.

For analysis of capsomeres produced from disassembled VLPs,
EDTA, EGTA, and DTT (final concentration 5 mM each) were added to the
5   sucrose cushions which were centrifuged at 250,000 x g for 2 to 4 hours at 4°C.
Fractions were collected by puncturing tubes from the bottom. A 1:10 dilution of
each fraction was then analyzed by antigen capture ELISA.

Example 4
Immunization Protocol for Production of
10 ### Polyclonal Antisera and Monoclonal Antibodies

Balb/c mice are immunized subcutaneously three times, every four
weeks with approximately 60 μg of HPV chimeric capsomeres mixed 1:1 with
complete or incomplete Freund's Adjuvants in a total volume of 100 μl. Six
weeks after the third immunization. mice are sacrificed and blood is collected by
15 cardiac puncture.

Example 5
Peptide ELISA to Quantitate Capsomere Formation

Microtiter plates (Dynatech) are coated overnight with 50 μl of
peptide E701 [Muller et al., 1982] at a concentration of 10 μg/ml in PBS. Wells
20 are blocked for 2 hour at 37°C with 100 μl of buffer containing 5% BSA and
0.05% Tween 20 in PBS and washed three times with PBS containing 0.05%
Tween 20. After the third wash. 50 μl of sera diluted 1:5000 in BSA/Tween
20/PBS is added to each well and incubation carried out for 1 hour. Plates are
washed again as before and 50 μl of goat-anti-mouse peroxidase conjugate is added
25 at a 1:5000 dilution. After 1 hour. plates are washed and stained using ABTS
substrate (0.2 mg/ml. 2.2'-Azino-bis(3-ethylbenzhiazoline-β-sulfonic acid in 0.1 M
Na-Acetate-Phosphate buffer (pH 4.2) with 4 μl 30% H_2O_2 per 10 ml).
Extinction is measured after 1 hour at 490 nm in a Dynatech automated plate
reader.

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Example 6
Antigen Capture ELISA to Quantitate Capsomere Formation

To allow relative quantification of virus-like particles and capsomeres in fractions of CsCl gradients, an antigen capture ELISA was utilized.

5 Microtiter plates were coated overnight with 50 μl/well of a 1:500 dilution (final concentration of 2 μg per ml, in PBS) with a protein A purified mouse monoclonal antibody immunospecific for HPV 16 L1 (antibodies 25/C, MM07 and Ritti 1 were obtained from mice immunized with HPV 16 VLPs). Plates were blocked with 5% milk/PBS for 1 hour and 50 μl of fractions of CsCl gradients were added

10 for 1 hour at 37°C using a 1:300 dilution (in 5% milk/PBS). After three washings with PBS/0.05% Tween 20, 50 μl of a polyclonal rabbit antiserum (1:3000 dilution in milk/PBS), raised against HPV 16 VLPs was added and plates were incubated at 37° for 1 hour. Plates were washed again and further incubated with 50 μl of a goat-anti-rabbit peroxidase conjugate (Sigma) diluted 1:5000 in PBS

15 containing 5% milk for 1 hour. After final washing, plates were stained with ABTS substrate for 30 minutes and extinction measured at 490 nm in a Dynatech automated plate reader. As a negative control, the assay also included wells coated only with PBS.

To test monoclonal antibodies for capsomere specificity, VLPs with

20 EDTA/DTT to disassemble particles. Treated particle preparations were assayed in the antigen-capture ELISA and readings compared to untreated controls. For disassembly, 40 μl of VLPs was incubated overnight at 4°C in 500 μl of disruption buffer containing 30 mM DTT, 50 mM EGTA, 60 mM EDTA, 100 mM NaCl, and 100 mM Tris/HCl, pH 8.0. Aliquots of treated and untreated

25 particles were used in the above capture ELISA in a 1:20-1:40 dilution.

Example 7
Hemagglutinin Inhibition Assay

In order to determine the extent to which chimeric capsomere vaccines evoke production of neutralizing antibodies, a hemagglutination inhibition

30 assay is carried out as briefly described below. This assay is based on previous

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observations that virus-like particles are capable of hemagglutinizing red blood cells.

Mice are immunized with any of a chimeric capsomere vaccine and sera is collected as described above in Example 4. As positive controls, HPV16
5   L1 virus like particles (VLPs) and bovine PV1 (BPV) L1 VLPs are assayed in parallel with a chimeric capsomere preparation. To establish a positive baseline, the HPV16 or BPV1 VLPs are first incubated with or without sera collected from immunized mice after which red blood cells are added. The extent to which preincubation with mouse cera inhibits red blood cell hemagglutinization is an
10   indication of the neutralizing capacity of the mouse sera. The experiments are then repeated using chimeric capsomeres in order to determine the neutralizing effect of the mouse sera on the vaccine. A brief protocol for the hemagglutination inhibition assay is described below.

One hundred microliters of heparin (1000 usp units/ml) are added to
15   1 ml fresh mouse blood. Red blood cells are washed three times with PBS followed by centrifugation and resuspension in a volume of 10 ml. Next, erythrocytes are resuspended in 0.5 ml PBS and stored at 4°C for up to three days. For the hemagglutinin assay. 70 μl of the suspension is used per well on a 96-well plate.

20   Chimeric capsomere aliquots from CsCl gradients are dialyzed for one hour against 10 mM Hepes (pH 7.5) and 100 μl of two-fold serial dilutions in PBS are added to mouse erythrocytes in round-bottom 96-well microtiter plates which are further incubated for 3-16 hours at 4°C. For hemagglutination inhibition, capsomeres are incubated with dilutions of antibodies in PBS for 60
25   minutes at room temperature and then added to the erythrocytes. The level of erythrocyte hemagglutination, and therefore the presence of neutralizing antibodies. is determined by standard methods.

In preliminary results. mouse sera generated against chimeric capsomeres comprising HPV16L1ΔC protein in association with E7 amino acid
30   residues 1-98 was observed to inhibit hemagglutination by HPV16 VLPs, but not

by BPV VLPs. The mouse sera was therefore positive for neutralizing antibodies against the human VLPs and this differential neutralization was most likely the result of antibody specificity for epitopes against which the antibodies were raised.

Numerous modifications and variations in the invention as set forth 5 in the above illustrative examples are expected to occur to those skilled in the art. Consequently only such limitations as appear in the appended claims should be placed on the invention.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: :

    (ii) TITLE OF INVENTION: Papilloma Virus Capsomere Vaccine Formulations and Methods of Use

    (iii) NUMBER OF SEQUENCES: 27

    (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: Marshall, O'Toole, Gerstein, Murray & Borun
        (B) STREET: 233 South Wacker Drive, 6300 Sears Tower
        (C) CITY: Chicago
        (D) STATE: Illinois
        (E) COUNTRY: United States of America
        (F) ZIP: 60606-6402

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

    (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: Williams Jr., Joseph A.
        (B) REGISTRATION NUMBER: 38,659
        (C) REFERENCE/DOCKET NUMBER: 27013/34028

    (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: 312-474-6300
        (B) TELEFAX: 312-474-0448

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1518 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (ix) FEATURE:
        (A) NAME/KEY: CDS
        (B) LOCATION: 1..1518

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ATG TCT CTT TGG CTG CCT AGT GAG GCC ACT GTC TAC TTG CCT CCT GTC       48
Met Ser Leu Trp Leu Pro Ser Glu Ala Thr Val Tyr Leu Pro Pro Val
1               5                   10                  15

CCA GTA TCT AAG GTT GTA AGC ACG GAT GAA TAT GTT GCA CGC ACA AAC       96
Pro Val Ser Lys Val Val Ser Thr Asp Glu Tyr Val Ala Arg Thr Asn
            20                  25                  30
```

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```
ATA TAT TAT CAT GCA GGA ACA TCC AGA CTA CTT GCA GTT GGA CAT CCC        144
Ile Tyr Tyr His Ala Gly Thr Ser Arg Leu Leu Ala Val Gly His Pro
        35                  40                  45

TAT TTT CCT ATT AAA AAA CCT AAC AAT AAC AAA ATA TTA GTT CCT AAA        192
Tyr Phe Pro Ile Lys Lys Pro Asn Asn Asn Lys Ile Leu Val Pro Lys
        50                  55                  60

GTA TCA GGA TTA CAA TAC AGG GTA TTT AGA ATA CAT TTA CCT GAC CCC        240
Val Ser Gly Leu Gln Tyr Arg Val Phe Arg Ile His Leu Pro Asp Pro
65                  70                  75                  80

AAT AAG TTT GGT TTT CCT GAC ACC TCA TTT TAT AAT CCA GAT ACA CAG        288
Asn Lys Phe Gly Phe Pro Asp Thr Ser Phe Tyr Asn Pro Asp Thr Gln
                85                  90                  95

CGG CTG GTT TGG GCC TGT GTA GGT GTT GAG GTA GGT CGT GGT CAG CCA        336
Arg Leu Val Trp Ala Cys Val Gly Val Glu Val Gly Arg Gly Gln Pro
                100                 105                 110

TTA GGT GTG GGC ATT AGT GGC CAT CCT TTA TTA AAT AAA TTG GAT GAC        384
Leu Gly Val Gly Ile Ser Gly His Pro Leu Leu Asn Lys Leu Asp Asp
                115                 120                 125

ACA GAA AAT GCT AGT GCT TAT GCA GCA AAT GCA GGT GTG GAT AAT AGA        432
Thr Glu Asn Ala Ser Ala Tyr Ala Ala Asn Ala Gly Val Asp Asn Arg
        130                 135                 140

GAA TGT ATA TCT ATG GAT TAC AAA CAA ACA CAA TTG TGT TTA ATT GGT        480
Glu Cys Ile Ser Met Asp Tyr Lys Gln Thr Gln Leu Cys Leu Ile Gly
145                 150                 155                 160

TGC AAA CCA CCT ATA GGG GAA CAC TGG GGC AAA GGA TCC CCA TGT ACC        528
Cys Lys Pro Pro Ile Gly Glu His Trp Gly Lys Gly Ser Pro Cys Thr
                165                 170                 175

AAT GTT GCA GTA AAT CCA GGT GAT TGT CCA CCA TTA GAG TTA ATA AAC        576
Asn Val Ala Val Asn Pro Gly Asp Cys Pro Pro Leu Glu Leu Ile Asn
                180                 185                 190

ACA GTT ATT CAG GAT GGT GAT ATG GTT GAT ACT GGC TTT GGT GCT ATG        624
Thr Val Ile Gln Asp Gly Asp Met Val Asp Thr Gly Phe Gly Ala Met
                195                 200                 205

GAC TTT ACT ACA TTA CAG GCT AAC AAA AGT GAA GTT CCA CTG GAT ATT        672
Asp Phe Thr Thr Leu Gln Ala Asn Lys Ser Glu Val Pro Leu Asp Ile
        210                 215                 220

TGT ACA TCT ATT TGC AAA TAT CCA GAT TAT ATT AAA ATG GTG TCA GAA        720
Cys Thr Ser Ile Cys Lys Tyr Pro Asp Tyr Ile Lys Met Val Ser Glu
225                 230                 235                 240

CCA TAT GGC GAC AGC TTA TTT TTT TAT TTA CGA AGG GAA CAA ATG TTT        768
Pro Tyr Gly Asp Ser Leu Phe Phe Tyr Leu Arg Arg Glu Gln Met Phe
                245                 250                 255

GTT AGA CAT TTA TTT AAT AGG GCT GGT GCT GTT GGT GAA AAT GTA CCA        816
Val Arg His Leu Phe Asn Arg Ala Gly Ala Val Gly Glu Asn Val Pro
                260                 265                 270

GAC GAT TTA TAC ATT AAA GGC TCT GGG TCT ACT GCA AAT TTA GCC AGT        864
Asp Asp Leu Tyr Ile Lys Gly Ser Gly Ser Thr Ala Asn Leu Ala Ser
        275                 280                 285
```

- 30 -

| TCA
Ser
290 | AAT
Asn | TAT
Tyr | TTT
Phe | CCT
Pro | ACA
Thr | CCT
Pro
295 | AGT
Ser | GGT
Gly | TCT
Ser | ATG
Met | GTT
Val
300 | ACC
Thr | TCT
Ser | GAT
Asp | GCC
Ala | 912 |

| CAA
Gln
305 | ATA
Ile | TTC
Phe | AAT
Asn | AAA
Lys | CCT
Pro | TAT
Tyr
310 | TGG
Trp | TTA
Leu | CAA
Gln | CGA
Arg | GCA
Ala
315 | CAG
Gln | GGC
Gly | CAC
His | AAT
Asn
320 | 960 |

| AAT
Asn | GGC
Gly | ATT
Ile | TGT
Cys | TGG
Trp
325 | GGT
Gly | AAC
Asn | CAA
Gln | CTA
Leu | TTT
Phe
330 | GTT
Val | ACT
Thr | GTT
Val | GTT
Val | GAT
Asp
335 | ACT
Thr | 1008 |

| ACA
Thr | CGC
Arg | AGT
Ser | ACA
Thr
340 | AAT
Asn | ATG
Met | TCA
Ser | TTA
Leu | TGT
Cys
345 | GCT
Ala | GCC
Ala | ATA
Ile | TCT
Ser | ACT
Thr
350 | TCA
Ser | GAA
Glu | 1056 |

| ACT
Thr | ACA
Thr | TAT
Tyr
355 | AAA
Lys | AAT
Asn | ACT
Thr | AAC
Asn | TTT
Phe
360 | AAG
Lys | GAG
Glu | TAC
Tyr | CTA
Leu | CGA
Arg
365 | CAT
His | GGG
Gly | GAG
Glu | 1104 |

| GAA
Glu | TAT
Tyr
370 | GAT
Asp | TTA
Leu | CAG
Gln | TTT
Phe | ATT
Ile
375 | TTT
Phe | CAA
Gln | CTG
Leu | TGC
Cys | AAA
Lys
380 | ATA
Ile | ACC
Thr | TTA
Leu | ACT
Thr | 1152 |

| GCA
Ala
385 | GAC
Asp | GTT
Val | ATG
Met | ACA
Thr | TAC
Tyr
390 | ATA
Ile | CAT
His | TCT
Ser | ATG
Met | AAT
Asn
395 | TCC
Ser | ACT
Thr | ATT
Ile | TTG
Leu | GAG
Glu
400 | 1200 |

| GAC
Asp | TGG
Trp | AAT
Asn | TTT
Phe | GGT
Gly
405 | CTA
Leu | CAA
Gln | CCT
Pro | CCC
Pro | CCA
Pro
410 | GGA
Gly | GGC
Gly | ACA
Thr | CTA
Leu | GAA
Glu
415 | GAT
Asp | 1248 |

| ACT
Thr | TAT
Tyr | AGG
Arg | TTT
Phe
420 | GTA
Val | ACC
Thr | TCC
Ser | CAG
Gln | GCA
Ala
425 | ATT
Ile | GCT
Ala | TGT
Cys | CAA
Gln | AAA
Lys
430 | CAT
His | ACA
Thr | 1296 |

| CCT
Pro | CCA
Pro | GCA
Ala
435 | CCT
Pro | AAA
Lys | GAA
Glu | GAT
Asp | CCC
Pro
440 | CTT
Leu | AAA
Lys | AAA
Lys | TAC
Tyr | ACT
Thr
445 | TTT
Phe | TGG
Trp | GAA
Glu | 1344 |

| GTA
Val | AAT
Asn | TTA
Leu | AAG
Lys
450 | GAA
Glu | AAG
Lys | TTT
Phe | TCT
Ser
455 | GCA
Ala | GAC
Asp | CTA
Leu | GAT
Asp | CAG
Gln
460 | TTT
Phe | CCT
Pro | TTA
Leu | 1392 |

| GGA
Gly
465 | CGC
Arg | AAA
Lys | TTT
Phe | TTA
Leu | CTA
Leu
470 | CAA
Gln | GCA
Ala | GGA
Gly | TTG
Leu | AAG
Lys
475 | GCC
Ala | AAA
Lys | CCA
Pro | AAA
Lys | TTT
Phe
480 | 1440 |

| ACA
Thr | TTA
Leu | GGA
Gly | AAA
Lys | CGA
Arg
485 | AAA
Lys | GCT
Ala | ACA
Thr | CCC
Pro | ACC
Thr
490 | ACC
Thr | TCA
Ser | TCT
Ser | ACC
Thr | TCT
Ser
495 | ACA
Thr | 1488 |

| ACT
Thr | GCT
Ala | AAA
Lys
500 | CGC
Arg | AAA
Lys | AAA
Lys | CGT
Arg | AAG
Lys | CTG
Leu
505 | TAA
* | | | | | | | 1518 |

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 506 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Ser Leu Trp Leu Pro Ser Glu Ala Thr Val Tyr Leu Pro Pro Val
 1           5               10              15

Pro Val Ser Lys Val Val Ser Thr Asp Glu Tyr Val Ala Arg Thr Asn
             20              25              30

Ile Tyr Tyr His Ala Gly Thr Ser Arg Leu Leu Ala Val Gly His Pro
         35              40              45

Tyr Phe Pro Ile Lys Lys Pro Asn Asn Asn Lys Ile Leu Val Pro Lys
     50              55              60

Val Ser Gly Leu Gln Tyr Arg Val Phe Arg Ile His Leu Pro Asp Pro
 65              70              75              80

Asn Lys Phe Gly Phe Pro Asp Thr Ser Phe Tyr Asn Pro Asp Thr Gln
             85              90              95

Arg Leu Val Trp Ala Cys Val Gly Val Glu Val Gly Arg Gly Gln Pro
         100             105             110

Leu Gly Val Gly Ile Ser Gly His Pro Leu Leu Asn Lys Leu Asp Asp
         115             120             125

Thr Glu Asn Ala Ser Ala Tyr Ala Ala Asn Ala Gly Val Asp Asn Arg
     130             135             140

Glu Cys Ile Ser Met Asp Tyr Lys Gln Thr Gln Leu Cys Leu Ile Gly
145             150             155             160

Cys Lys Pro Pro Ile Gly Glu His Trp Gly Lys Gly Ser Pro Cys Thr
             165             170             175

Asn Val Ala Val Asn Pro Gly Asp Cys Pro Pro Leu Glu Leu Ile Asn
         180             185             190

Thr Val Ile Gln Asp Gly Asp Met Val Asp Thr Gly Phe Gly Ala Met
     195             200             205

Asp Phe Thr Thr Leu Gln Ala Asn Lys Ser Glu Val Pro Leu Asp Ile
     210             215             220

Cys Thr Ser Ile Cys Lys Tyr Pro Asp Tyr Ile Lys Met Val Ser Glu
225             230             235             240

Pro Tyr Gly Asp Ser Leu Phe Phe Tyr Leu Arg Arg Glu Gln Met Phe
             245             250             255

Val Arg His Leu Phe Asn Arg Ala Gly Ala Val Gly Glu Asn Val Pro
         260             265             270

Asp Asp Leu Tyr Ile Lys Gly Ser Gly Ser Thr Ala Asn Leu Ala Ser
     275             280             285

Ser Asn Tyr Phe Pro Thr Pro Ser Gly Ser Met Val Thr Ser Asp Ala
     290             295             300
```

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```
Gln Ile Phe Asn Lys Pro Tyr Trp Leu Gln Arg Ala Gln Gly His Asn
305             310         315             320

Asn Gly Ile Cys Trp Gly Asn Gln Leu Phe Val Thr Val Val Asp Thr
                325             330             335

Thr Arg Ser Thr Asn Met Ser Leu Cys Ala Ala Ile Ser Thr Ser Glu
            340             345             350

Thr Thr Tyr Lys Asn Thr Asn Phe Lys Glu Tyr Leu Arg His Gly Glu
        355             360             365

Glu Tyr Asp Leu Gln Phe Ile Phe Gln Leu Cys Lys Ile Thr Leu Thr
    370             375             380

Ala Asp Val Met Thr Tyr Ile His Ser Met Asn Ser Thr Ile Leu Glu
385             390             395             400

Asp Trp Asn Phe Gly Leu Gln Pro Pro Pro Gly Gly Thr Leu Glu Asp
                405             410             415

Thr Tyr Arg Phe Val Thr Ser Gln Ala Ile Ala Cys Gln Lys His Thr
            420             425             430

Pro Pro Ala Pro Lys Glu Asp Pro Leu Lys Lys Tyr Thr Phe Trp Glu
        435             440             445

Val Asn Leu Lys Glu Lys Phe Ser Ala Asp Leu Asp Gln Phe Pro Leu
    450             455             460

Gly Arg Lys Phe Leu Leu Gln Ala Gly Leu Lys Ala Lys Pro Lys Phe
465             470             475             480

Thr Leu Gly Lys Arg Lys Ala Thr Pro Thr Thr Ser Ser Thr Ser Thr
            485             490             495

Thr Ala Lys Arg Lys Lys Arg Lys Leu    *
            500             505
```

    (2) INFORMATION FOR SEQ ID NO:3:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 297 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA

        (ix) FEATURE:
            (A) NAME/KEY: CDS
            (B) LOCATION: 1..297

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ATG CAT GGA GAT ACA CCT ACA TTG CAT GAA TAT ATG TTA GAT TTG CAA    48
Met His Gly Asp Thr Pro Thr Leu His Glu Tyr Met Leu Asp Leu Gln
1               5                   10                  15

CCA GAG ACA ACT GAT CTC TAC TGT TAT GAG CAA TTA AAT GAC AGC TCA    96
Pro Glu Thr Thr Asp Leu Tyr Cys Tyr Glu Gln Leu Asn Asp Ser Ser
            20                  25                  30
```

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```
GAG GAG GAG GAT GAA ATA GAT GGT CCA GCT GGA CAA GCA GAA CCG GAC      144
Glu Glu Glu Asp Glu Ile Asp Gly Pro Ala Gly Gln Ala Glu Pro Asp
        35              40                45

AGA GCC CAT TAC AAT ATT GTA ACC TTT TGT TGC AAG TGT GAC TCT ACG      192
Arg Ala His Tyr Asn Ile Val Thr Phe Cys Cys Lys Cys Asp Ser Thr
    50              55              60

CTT CGG TTG TGC GTA CAA AGC ACA CAC GTA GAC ATT CGT ACT TTG GAA      240
Leu Arg Leu Cys Val Gln Ser Thr His Val Asp Ile Arg Thr Leu Glu
65              70              75                  80

GAC CTG TTA ATG GGC ACA CTA GGA ATT GTG TGC CCC ATC TGT TCT CAG      288
Asp Leu Leu Met Gly Thr Leu Gly Ile Val Cys Pro Ile Cys Ser Gln
                85              90                  95

AAA CCA TAA                                                          297
Lys Pro  *
```

(2) INFORMATION FOR SEQ ID NO:4:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 98 amino acids
             (B) TYPE: amino acid
             (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met His Gly Asp Thr Pro Thr Leu His Glu Tyr Met Leu Asp Leu Gln
 1              5               10              15

Pro Glu Thr Thr Asp Leu Tyr Cys Tyr Glu Gln Leu Asn Asp Ser Ser
            20              25              30

Glu Glu Glu Asp Glu Ile Asp Gly Pro Ala Gly Gln Ala Glu Pro Asp
        35              40                45

Arg Ala His Tyr Asn Ile Val Thr Phe Cys Cys Lys Cys Asp Ser Thr
    50              55              60

Leu Arg Leu Cys Val Gln Ser Thr His Val Asp Ile Arg Thr Leu Glu
65              70              75                  80

Asp Leu Leu Met Gly Thr Leu Gly Ile Val Cys Pro Ile Cys Ser Gln
                85              90                  95

Lys Pro  *
```

(2) INFORMATION FOR SEQ ID NO:5:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 34 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

- 34 -

CCCCGATATC GCCTTTAATG TATAAATCGT CTGG   34

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 35 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCCGATATC TCAAATTATT TTCCTACACC TAGTG   35

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 40 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AAAGATATCT TGTAGTAAAA ATTTGCGTCC TAAAGGAAAC   40

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 44 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AAAGATATCT AATCTACCTC TACAACTGCT AAACGCAAAA AACG   44

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 35 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AAAAGATATC ATGCATGGAG ATACACCTAC ATTGC   35

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 34 base pairs

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```
        (B)  TYPE:  nucleic acid
        (C)  STRANDEDNESS:  single
        (D)  TOPOLOGY:  linear

   (ii)  MOLECULE TYPE:  DNA

   (xi)  SEQUENCE DESCRIPTION:  SEQ ID NO:10:
```

TTTTGATATC GGCTCTGTCC GGTTCTGCTT GTCC   34

(2) INFORMATION FOR SEQ ID NO:11:

```
   (i)  SEQUENCE CHARACTERISTICS:
        (A)  LENGTH:  44 base pairs
        (B)  TYPE:  nucleic acid
        (C)  STRANDEDNESS:  single
        (D)  TOPOLOGY:  linear

   (ii)  MOLECULE TYPE:  DNA

   (xi)  SEQUENCE DESCRIPTION:  SEQ ID NO:11:
```

TTTTGATATC CTTGCAACAA AAGGTTACAA TATTGTAATG GGCC   44

(2) INFORMATION FOR SEQ ID NO:12:

```
   (i)  SEQUENCE CHARACTERISTICS:
        (A)  LENGTH:  35 base pairs
        (B)  TYPE:  nucleic acid
        (C)  STRANDEDNESS:  single
        (D)  TOPOLOGY:  linear

   (ii)  MOLECULE TYPE:  DNA

   (xi)  SEQUENCE DESCRIPTION:  SEQ ID NO:12:
```

AAAAGATATC TGGTTTCTGA GAACAGATGG GGCAC   35

(2) INFORMATION FOR SEQ ID NO:13:

```
   (i)  SEQUENCE CHARACTERISTICS:
        (A)  LENGTH:  38 base pairs
        (B)  TYPE:  nucleic acid
        (C)  STRANDEDNESS:  single
        (D)  TOPOLOGY:  linear

   (ii)  MOLECULE TYPE:  DNA

   (xi)  SEQUENCE DESCRIPTION:  SEQ ID NO:13:
```

TTTTGATATC GATTATGAGC AATTAAATGA CAGCTCAG   38

(2) INFORMATION FOR SEQ ID NO:14:

```
   (i)  SEQUENCE CHARACTERISTICS:
        (A)  LENGTH:  35 base pairs
        (B)  TYPE:  nucleic acid
        (C)  STRANDEDNESS:  single
        (D)  TOPOLOGY:  linear

   (ii)  MOLECULE TYPE:  DNA

   (xi)  SEQUENCE DESCRIPTION:  SEQ ID NO:14:
```

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TTTTGATATC GTCTACGTGT GTGCTTTGTA CGCAC   35

(2) INFORMATION FOR SEQ ID NO:15:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 39 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TTTATCGATA TCGGTCCAGC TGGACAAGCA GAACCGGAC   39

(2) INFORMATION FOR SEQ ID NO:16:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 39 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TTTTGATATC GATGCCCATT ACAATATTGT AACCTTTTG   39

(2) INFORMATION FOR SEQ ID NO:17:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 294 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA

        (ix) FEATURE:
            (A) NAME/KEY: CDS
            (B) LOCATION: 1..294

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ATG AGT CTT CTA ACC GAG GTC GAA ACG CTT ACC AGA AAC GGA TGG GAG   48
Met Ser Leu Leu Thr Glu Val Glu Thr Leu Thr Arg Asn Gly Trp Glu
 1   5   10   15

TGC AAA TGC AGC GAT TCA AGT GAT CCT CTC ATT ATC GCA GCG AGT ATC   96
Cys Lys Cys Ser Asp Ser Ser Asp Pro Leu Ile Ile Ala Ala Ser Ile
             20   25   30

ATT GGG ATC TTG CAC TTG ATA TTG TGG ATT TTT TAT CGT CTT TTC TTC   144
Ile Gly Ile Leu His Leu Ile Leu Trp Ile Phe Tyr Arg Leu Phe Phe
             35   40   45

AAA TGC ATT TAT CGT CGC CTT AAA TAC GGT TTG AAA AGA GGG CCT TCT   192
Lys Cys Ile Tyr Arg Arg Leu Lys Tyr Gly Leu Lys Arg Gly Pro Ser
             50   55   60

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```
ACG GAA GGA GCG CCT GAG TCT ATG AGG GAA GAA TAT CGG CAG GAA CAG    240
Thr Glu Gly Ala Pro Glu Ser Met Arg Glu Glu Tyr Arg Gln Glu Gln
65              70              75              80

CAG AGT GCT GTG GAT GTT GAC GAT GTT CAT TTT GTC AAC ATA GAG CTG    288
Gln Ser Ala Val Asp Val Asp Asp Val His Phe Val Asn Ile Glu Leu
                85              90              95

GAG TAA                                                            294
Glu *
```

(2) INFORMATION FOR SEQ ID NO:18:

       (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 97 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: protein

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Met Ser Leu Leu Thr Glu Val Glu Thr Leu Thr Arg Asn Gly Trp Glu
1               5               10              15

Cys Lys Cys Ser Asp Ser Ser Asp Pro Leu Ile Ile Ala Ala Ser Ile
            20              25              30

Ile Gly Ile Leu His Leu Ile Leu Trp Ile Phe Tyr Arg Leu Phe Phe
        35              40              45

Lys Cys Ile Tyr Arg Arg Leu Lys Tyr Gly Leu Lys Arg Gly Pro Ser
    50              55              60

Thr Glu Gly Ala Pro Glu Ser Met Arg Glu Glu Tyr Arg Gln Glu Gln
65              70              75              80

Gln Ser Ala Val Asp Val Asp Asp Val His Phe Val Asn Ile Glu Leu
            85              90              95

Glu *
```

(2) INFORMATION FOR SEQ ID NO:19:

       (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 40 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
TTTTGATATC GATATGGAAT GGCTAAAGAC AAGACCAATC                         40
```

(2) INFORMATION FOR SEQ ID NO:20:

       (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 35 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single

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        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TTTTGATATC GTTGTTTGGA TCCCCATTCC CATTG   35

(2) INFORMATION FOR SEQ ID NO:21:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 24 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GTTATGACAT ACATACATTC TATG   24

(2) INFORMATION FOR SEQ ID NO:22:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 35 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CCATGCATTC CTGCTTGTAG TAAAAATTTG CGTCC   35

(2) INFORMATION FOR SEQ ID NO:23:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 29 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTACAAGCAG GAATGCATGG AGATACACC   29

(2) INFORMATION FOR SEQ ID NO:24:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 36 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CATCTGAAGC TTAGTAATGG GCTCTGTCCG GTTCTG   36

- 39 -

(2) INFORMATION FOR SEQ ID NO:25:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 38 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CATCTGAAGC TTATCAATAT TGTAATGGGC TCTGTCCG   38

(2) INFORMATION FOR SEQ ID NO:26:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 54 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CATCTGAAGC TTACTTGCAA CAAAAGGTTA CAATATTGTA ATGGGCTCTG TCCG   54

(2) INFORMATION FOR SEQ ID NO:27:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 69 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CATCTGAAGC TTAAAGCGTA GAGTCACACT TGCAACAAAA GGTTACAATA TTGTAATGGG   60

CTCTGTCCG   69

(2) INFORMATION FOR SEQ ID NO:28:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 47 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CATCTGAAGC TTATTGTACG CACAACCGAA GCGTAGAGTC ACACTTG   47

- 40 -

WHAT IS CLAIMED IS:

1.   A vaccine formulation comprising a human papilloma virus capsomere, said capsomere comprising a fusion protein comprising a human papilloma virus L1 protein adjacent amino acid residues from a second protein.

2.   A vaccine formulation comprising a human papilloma virus capsomere. said capsomere comprising a truncated human papilloma virus L1 protein having a deletion of one or more amino acid residues necessary for formation of a virus-like particle.

3.   The vaccine formulation of claim 2 wherein said capsomere comprises a fusion protein comprising a truncated human papilloma virus L1 protein adjacent amino acid residues from a second protein.

4.   The vaccine formulation of any one of claims 1,2, or 3 wherein the L1 protein is encoded in the genome of a human papilloma virus selected from the group consisting of HPV6. HPV11. HPV16, HPV18. HPV33. HPV35. and HPV45.

5.   The vaccine formulation of claim 4 wherein the papilloma virus is HVP16.

6.   The vaccine formulation of any one of claims 2, 3, or 5 wherein carboxy terminal amino acid residues are deleted from the L1 protein.

7.   The vaccine formulation of claim 6 wherein 1 to 34 carboxy terminal amino acid residues are deleted from the L1 protein.

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8.   The vaccine formulation of claim 7 wherein 34 carboxy terminal amino acid residues are deleted from the L1 protein.

9.   The vaccine formulation of any one of claims 2, 3, or 5 wherein amino terminal amino acid residues are deleted from the L1 protein.

10.   The vaccine formulation of any one of claims 2, 3, or 5 wherein internal amino acid residues are deleted from the L1 protein.

11.   The vaccine formulation of claim 10 wherein the amino acid residues deleted from the L1 protein comprise a nuclear localization signal.

12.   The vaccine formulation of claims 2 or 3 wherein the amino acids residues from the second protein are derived from an HPV protein.

13.   The vaccine formulation of claim 12 wherein the HPV protein is an early HPV protein.

14.   The vaccine formulation of claim 12 wherein the early HPV protein is selected from the group consisting of E1, E2, E3, E4. E5. E6. and E7.

15.   A method of treating an individual infected with an HPV virus comprising the step of administering to a patient in need thereof an amount of the vaccine formulation of claims 1, 2, 3, 5, 7, 8, 11, 13 or 14 effective to reduce the level of HPV infection.

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16.   A method for preventing papilloma virus infection comprising the step of administering to an individual susceptible thereto an amount of the vaccine formulation of claims 1, 2, 3, 5, 7, 8, 11, 13 or 14 effective to inhibit HPV infection.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/62   A61K39/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication. where appropriate, of the relevant passages	Relevant to claim No.
X	MÜLLER M ET AL.: "Chimeric papillomavirus-like particles" VIROLOGY, vol. 234, no. 1, 21 July 1997, pages 93-111, XP002091857 ORLANDO US see the whole document ---	1-8, 10-16
X	DE 44 35 907 A (GISSMANN L;ZHOU J; MÜLLER M) 11 April 1996 see the whole document ---	1-5, 10-16
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 February 1999	16/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cupido, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document. with indication.where appropriate. of the relevant passages	Relevant to claim No.
X	LI M ET AL.: "Expression of the human papillomavirus type 11 L1 capsid protein in Escherichia coli" JOURNAL OF VIROLOGY, vol. 71, no. 4, April 1997, pages 2988-2995, XP002091858 AMERICAN SOCIETY FOR MICROBIOLOGY US see figure 7	1-4,9
A	PAINTSIL J ET AL.: "Carboxy terminus of bovine pappilomavirus type-1 L1 protein is not required for capsid formation" VIROLOGY, vol. 223, no. 1, 1 September 1996, pages 238-244, XP002091859 ORLANDO US see figure 1	1-16
A	ROSE R C ET AL: "SEROLOGICAL DIFFERENTIATION OF HUMAN PAPILLOMAVIRUS TYPES 11, 16 AND 18 USING RECOMBINANT VIRUS-LIKE PARTICLES" JOURNAL OF GENERAL VIROLOGY, vol. 75, no. 9, September 1904, pages 2445-2449, XP000604635	1-16
A	WO 96 11274 A (US DEPARTMENT OF HEALTH) 18 April 1996 see examples 1-7	1-16

INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were f und unsearchable (Continuation of item 1 f first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

   Remark: Although claims 15 and 16
   are directed to a method of treatment of the human or animal
   body, the search has been carried out and based on the alleged
   effects of the vaccine formulation.

2. [ ] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such
   an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all
   searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report
   covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

                               [ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

Inter   rnal Application No
PCT/US 98/20965

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
DE 4435907	A	11-04-1996	AU	4270196 A	02-05-1996
			CA	2202090 A	18-04-1996
			WO	9611272 A	18-04-1996
			EP	0809700 A	03-12-1997
WO 9611274	A	18-04-1996	US	5618536 A	08-04-1997
			AU	3828495 A	02-05-1996
			EP	0789766 A	20-08-1997
			JP	10506796 T	07-07-1998
			US	5855891 A	05-01-1999

Chimeric papillomavirus virus-like particles elicit antitumor immunity against the E7 oncoprotein in an HPV16 tumor model

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ABSTRACT   Papillomavirus-like particles (VLPs) are a promising prophylactic vaccine candidate to prevent human papillomavirus (HPV) infections and associated epithelial neoplasia. However, they are unlikely to have therapeutic effects because the virion capsid proteins are not detected in the proliferating cells of the infected epithelia or in cervical carcinomas. To increase the number of viral antigen targets for cell-mediated immune responses in a VLP-based vaccine, we have generated stable chimeric VLPs consisting of the L1 major capsid protein plus the entire E7 (11 kDa) or E2 (43 kDa) nonstructural papillomavirus protein fused to the L2 minor capsid protein. The chimeric VLPs are indistinguishable from the parental VLPs in their morphology and in their ability to agglutinate erythrocytes and elicit high titers of neutralizing antibodies. Protection from tumor challenge was tested in C57BL/6 mice by using the tumor cell line TC-1, which expresses HPV16 E7, but not the virion structural proteins. Injection of HPV16 L1/L2-HPV16 E7 chimeric VLPs, but not HPV16 L1/L2 VLPs, protected the mice from tumor challenge, even in the absence of adjuvant. The chimeric VLPs also induced protection against tumor challenge in major histocompatibility class II-deficient mice, but not in β_2-microglobulin or perforin knockout mice implying that protection was mediated by class I-restricted cytotoxic lymphocytes. These findings raise the possibility that VLPs may generally be efficient vehicles for generating cell-mediated immune responses and that, specifically, chimeric VLPs containing papillomavirus nonstructural proteins may increase the therapeutic potential of VLP-based prophylactic vaccines in humans.

Human papillomaviruses (HPVs) that infect the genital tract are associated with human anogenital tract cancer, particularly cervical cancer (reviewed in ref. 1). HPVs are thought to be the primary causative agent in >90% of cervical cancers (2), with HPV16 being the type most frequently found in these tumors. Approximately 500,000 women develop cervical cancer each year, and 200,000 women die from it, making this disease the second-most common cause of cancer deaths in women worldwide (3).

Significant advances have been made recently in the development of a candidate prophylactic vaccine against papillomavirus infections (reviewed in ref. 4). Expression of the papillomavirus major capsid protein, L1, in eukaryotic cells leads to self-assembly into virus-like particles (VLPs) that are morphologically indistinguishable from native virions and present the conformational epitopes required for the induction of high titer neutralizing antisera (5). L2, the minor capsid

protein, coassembles with L1 at a ratio of ≈1 L2 molecule to 30 L1 molecules (6). Although L2 presents some epitopes that induce the production of neutralizing antiserum (7), most neutralizing antibodies induced by L1/L2 VLPs recognize L1 determinants (8). Several studies have shown that L1 and L1/L2 VLP-based vaccines protect animals against high dose experimental papillomavirus infection (9–11). Protection was passively transferred by the sera of immunized animals, indicating that neutralizing antibodies were sufficient to confer protection (9, 11). The initial clinical trials of HPV VLP-based vaccines in humans are now under way (12).

It is unlikely that cell-mediated responses to L1 or L1/E2 VLPs would have a significant therapeutic effect against established papillomaviral infections. This speculation is based on the observation that L1 and L2 proteins are undetectable in the most likely targets of immune regression: the basal epithelial cells of benign productive lesions and the abnormal proliferative cells in premalignant and malignant lesions (13). In contrast, other viral genes, such as E7 or E2, are likely to be expressed in these cells. Therefore these proteins are potential targets for cell-mediated immune regression.

In an effort to generate an HPV vaccine candidate with both improved prophylactic and, in addition, therapeutic potential, we have generated chimeric VLPs containing L1 and fusion proteins consisting of L2 linked to another papillomavirus protein. We chose L2 as the fusion partner because it is not required for capsid assembly or cell surface interactions (14, 15). Therefore larger and more varied insertions might be compatible with normal assembly and cell binding, in comparison with L1 chimeras. In addition, we did not wish to adversely affect the immunodominant conformational neutralizing epitopes on L1. We have generated HPV16 E7 chimeras because the 11-kDa E7 protein is selectively expressed at relatively high levels in high grade cervical dysplasias and cancers (13), and mouse tumor vaccine models have been developed for this protein (16–18). We also show that chimeric VLPs can be made with L2 fused to a 43-kDa full-length viral E2 protein.

MATERIALS AND METHODS

Generation of HPV16 L2 Chimeric Recombinant Baculoviruses. Two HPV16 E7 chimeras were generated: 16L2-16E7 and BL2-16E7. They each contain the full-length HPV16 E7 gene (coding for 98 aa) fused to the 3' end of the full-length HPV16 L2 (473 aa) or BPV1 L2 (469 aa) gene, respectively.

Medical Sciences: Greenstone *et al.*

Proc. Natl. Acad. Sci. USA 95 (1998)   1801

The chimeras were generated via recombinant PCR (19) whereby L2 was amplified with a 5' oligonucleotide containing a restriction enzyme site, and a 3' oligonucleotide that was complementary to the E7 5' oligonucleotide. E7 was then amplified with a 5' oligonucleotide complementary to the L2 3' oligonucleotide and a 3' oligonucleotide containing a restriction enzyme site. The L2 and E7 genes were then fused in a second primer extension reaction by using only the outside (L2 5', and E7 3') oligonucleotides. The fused L2-E7 genes were then cloned immediately downstream of the pSyn promoter into the baculovirus double expression vector pSyn-wtVI⁻, which already contained the respective L1 genes cloned under the polyhedrin promoter (6). BL2–16E7 was cloned as a 5' BglII to 3' BglII fragment, and primers used were as follows (restriction sites are underlined): BL2, sense, 5'-GCGGTAG-ATCTACCTATAAATATGAGTGCACGAAAAAGAGT-AAAACGT-3', and antisense, 5'-GCAATGTAGGTGTAT-CTCCATGCATGGCATGTTTCCGTTTTTTTCGTTTCC-TCAACAAGGAGGG-3'; HPV16E7, sense, 5'-CCCTCCTT-GTTGAGGAAACGAAAAAAACGGAAACATGCCAT-GCATGGAGATACACCTACATTGC-3' and antisense 5'-CCGCTAGATCTGGTACCTGCAGGATCAGCCATG-G-3'. 16L2-16E7 was cloned as a 5' SstII to 3' SstII fragment, and the primers used were as follows (restriction sites are underlined): HPV16L2, sense, 5'-GCGGTCCGCGGAATA-TGCGACACAAACGTTCTGCAAAACGCACAAAACG-T-3' and antisense, 5'-ATCTCCATGCATGGCAGCCAAA-GAGAC-3'; HPV16E7, sense, 5'-GTCTCTTTGGCTGCCA-TGCATGGAGAT-3' and antisense, 5'-GCTCCGCGGG-GTACCTGCAGGATCAGCC-3'.

Recombinant baculovirus stocks were generated by cotransfection with baculovirus DNA (Baculo-Gold; PharMingen) by using lipofectin (GIBCO/BRL). Plaque purification was performed by using published techniques (5).

An L2-E2 chimera was also generated by fusing the coding sequence of the full-length cottontail rabbit papillomavirus (CRPV) E2 (391 aa) to the C-terminal amino acid of HPV16 L2. This chimera (16L2-CE2) was generated by simultaneously ligating L2 and E2 into the baculovirus expression vector, pFastBac1 (GIBCO/BRL). An XhoI site was inserted into the sense L2 primer, and a KpnI site was used in the antisense E2 primer for cloning into pFastBac1. An SstII site was used to fuse the two genes. The primers used were as follows (restriction sites are underlined): 16L2 sense, 5'-CCGCTCGAGAA-TATGCGACACAAACGTTCT-3'; 16L2 antisense, 5'-TC-CCCGCGGGGCAGCCAAAGAGACATC-3' and CE2 sense, 5'-GGCGCCGCGGATGGAGGCTCTCAGCCAGC-G-3'; CE2 antisense, 5'-GGCGGGTACCGCTGCTGATGG-GAATGGG-3'.

Recombinant 16L2-CE2 baculoviruses were generated by using the Bac-to-Bac system according to the manufacturer's instructions (GIBCO/BRL).

Coexpression of Chimeric L2 Proteins with L1 in Insect Cells. Sf9 cells were mock infected or infected at a multiplicity of infection (moi) of ≈10 with either wild-type L1/L2 or L1/L2-E7 chimeric recombinant baculoviruses. For generation of 16L1/L2-CE2, Sf9 cells were coinfected with the 16L2-CE2 baculoviruses and baculoviruses containing the gene for HPV16 L1 (6) at an moi of ≈5 for each. After 72 h, cells were lysed by boiling in SDS sample buffer and analyzed by SDS/PAGE in 10% gels. Proteins were stained with 0.25% Coomassie blue or analyzed by Western blotting with HPV16 L1-VLP (6), GST-HPV16 L2 (6), BPV1 L1-VLP (5), GST-BPV1 L2 (7), trp-HPV16 E7 (20) rabbit antisera, or an anti-HPV16 E7 mAb (CIBA-Corning, Alameda, CA) (GST, glutathione S-transferase).

Purification of Chimeric VLPs and Transmission Electron Microscopy (TEM). After purification by CsCl gradient centrifugation as described (6), particles were adsorbed to carbon-coated grids, stained with 1% uranyl acetate, and examined

with a Philips electron microscope model EM 400RT at ×36,000 magnification.

Coimmunoprecipitation of L1/L2-E7 Complexes. CsCl gradient-purified chimeric VLPs were immunoprecipitated in PBS, 1% Triton X-100 with anti-L1 mAb 5B6 for the bovine papillomavirus (BPV) VLPs (7), mAb H16.V5 for the HPV16 VLPs (kindly provided by N. Christensen) (21), or an irrelevant antibody (anti-E1A mAb) and protein A-Sepharose and subjected to SDS/PAGE. Proteins were immunoblotted and the blots were probed with rabbit antisera to trp-HPV16 E7 and GST-BPVL2 or GST-HPV16L2 for the BPV and HPV16 VLPs containing the L2-E7 chimeras, respectively. The blots of the 16L2-CE2 chimeric VLPs were probed with anti-GST-HPV16L2. The blots were probed with goat, anti-rabbit horse-radish peroxidase-conjugated antiserum and developed via chemiluminescence (Kirkegaard & Perry Laboratories).

Chimeric VLP Antisera. Rabbits were immunized by s.c. injection with three doses of 300 μg of CsCl gradient-purified VLPs in PBS (native) or VLPs boiled for 5 min in PBS/1% SDS (denatured) as described (6).

To determine the L1 or E7-specific antibody titers of VLP-immunized mice, 0.5 μg of purified papillomavirus VLPs, or 6-histidine tagged (6-His) E7 (R. M. Melillo, personal communication), was bound to each well of a 96-well plate, and ELISAs were performed by using a mouse-hybridoma subtyping kit (Boehringer Mannheim). Polyomavirus VP1 VLPs (VP1-baculovirus kindly provided by R. Garcea) (22), or 6-His chloramphenicol acetyltransferase (GIBCO/BRL) were bound to plates and served as negative controls for the papillomavirus VLPs or 6-His E7, respectively. The sera were tested at dilutions from 1:100 to 1:100,000. Titers were determined by subtracting averaged readings of sera from PBS-injected (naive) mice at each dilution from the mean readings of sera from immunized mice ($n = 5$) at the same dilution. Reported titers were those found to be >0.2 OD_{405nm} unit over that of titers obtained from the plates bound with the negative control proteins.

Cell Lines and Mice. The TC-1 cell line was generated by transduction of C57BL/6 (B6) primary lung epithelial cells with a retroviral vector expressing HPV16 E6/E7 plus a retrovirus expressing activated c-Ha-*ras* (16). B6 mice were obtained from The Jackson Laboratory and were used at 7–10 weeks of age. B6 mice with germ-line disruption of β_2-microglobulin, perforin, or major histocompatibility complex (MHC) class II expression have been described (23–25).

Tumor Challenge. Mice were immunized by s.c. injection with 140 μg VLPs in PBS and boosted once 3 weeks later with 10 μg VLPs or by a single inoculation of 10 μg VLPs. The mice were challenged 2 weeks later with 2 × 10⁴ TC-1 tumor cells administered s.c. Tumor take was assessed on day 45. Natural killer (NK) cell depletion was accomplished by injection of monoclonal anti-NK1.1 isolated from supernatants of the PK136 hybridoma (American Type Culture Collection) as described (26). Differences in the results of the tumor challenge assays were evaluated by the two-tailed Fisher's exact test by using SAS software release 6.12.

RESULTS

To generate a candidate vaccine that contained as many HPV16 E7 epitopes as possible, we attempted to incorporate the entire HPV16 E7 protein into papillomavirus VLPs. We constructed L2-E7 fusions by using the L2 of HPV16 or BPV1, and coexpressed the chimeras with the homologous L1 genes in Sf9 insect cells via double recombinant baculoviruses. E7 was fused to the C terminus of L2 because, except for several basic amino acids, this portion of L2 is poorly conserved among papillomaviruses. The 16L1/L2–16E7 and BL1/L2–16E7 chimeric VLPs were predicted to consist of the L1 protein (506 aa for HPV16 and 496 aa for BPV1) plus a full-length HPV16E7 (98 aa) fused to the C-terminal end of the entire L2

protein (473 aa and 469 aa for HPV16 and BPV, respectively). The L2 fusions and the corresponding L1 proteins were coexpressed in Sf9 insect cells, leading to the appearance of L2, E7 and L1 immunoreactive species of the predicted molecular weight on immunoblots of extracts of the infected cells (Fig. 1 and data not shown).

Coassembly of L2-E7 into VLPs. To obtain evidence for the formation of a stable complex between L1 and the L2-E7 chimera, we immunoprecipitated chimeric BPV and HPV16 VLPs with the appropriate polyclonal or mAb to L1 and analyzed the precipitated complexes by SDS/PAGE and immunoblotting with L2 and E7 antibodies. L1 antibodies, but not the preimmune sera or a control mAb, were able to precipitate a complex that contained the expected molecular weight immunoreactive L2 and HPV16 E7 species from the chimeric VLP preparations, but not from control L1 alone or L1/L2 VLPs (Fig. 1*A*, data shown for HPV VLPs). These results established that the L2-E7 fusion proteins form a stable complex with L1 when coexpressed in insect cells. By using equivalent amounts of L1 protein, similar levels of L2 and the L2-E7 fusion protein were coimmunoprecipitated from parental and chimeric VLPs, respectively, with an L1 to L2 ratio of approximately 30:1 (Fig. 1 and data not shown).

To examine VLP morphology, VLP preparations were purified by centrifugation through a 40% sucrose cushion and banded in a CsCl density gradient. By TEM, negatively stained samples of both the chimeric HPV16 and BPV VLPs were found to contain similarly large numbers of particles that appeared morphologically indistinguishable from the parental L1/L2 VLPs (Fig. 2; data shown for HPV VLPs).

Cosedimentation analysis was performed to obtain additional evidence that the chimeric L2s were coassembling with L1 into the VLPs. After purification over two sequential CsCl density gradients, VLP preparations were analyzed by analytical sucrose gradient centrifugation. The composition of the resulting gradient fractions was determined by Coomassie-stained SDS/PAGE and Western blot analysis with antibodies specific for L1, L2, or E7, as well as by electron microscopy. The density profiles of chimeric and L1/L2 VLP gradients were very similar as determined by the refractive index (data not shown). Consistent with the combined masses of L2 and E7, we detected a band of ≈100 kDa that was specifically recognized by both the L2 and E7 antisera in a dense fraction of the gradient (Fig. 3, rows B and C, fraction 10, data shown for BPV VLPs). An E7-reactive band was not detected in gradients of VLPs consisting of only L1 and L2, although the expected L2 immunoreactive protein with an apparent molecular mass of 70 kDa was detected in fraction 10 (data not shown). This same fraction (lane 10) contained the majority of

FIG. 2. Chimeric VLPs containing the L2-E7 or L2-E2 fusion proteins retain the ability to self-assemble into well-formed particles. VLP preparations were stained with uranyl acetate and examined by TEM. (*A*) 16L1/L2. (*B*) 16L1/L2-16E7. (*C*) 16L1/L2-CE2. (×36,000; bar = 100 nm.)

the L1 protein, as determined by Coomassie-stained SDS/PAGE (Fig. 3, row A) and the majority of the well-formed VLPs as determined by TEM (data not shown). From these results, we conclude that the L2-E7 fusion proteins are incorporated into VLPs when coexpressed with L1 in insect cells. Rabbit antisera to the BL1/L2-16E7 chimeric VLPs recognized only one (peptide B) of six overlapping L2 peptides (A–F) that together spanned the entire L2 protein (data not shown). Because, in a previous study (7), this L2 peptide was found to have epitopes on the surface of infectious virions, these results support the conclusion that the L2 chimera is positioned normally in the chimeric VLPs.

Surface Properties of Chimeric VLPs. Papillomavirus virions bind avidly to cell surfaces (15). Because this feature might potentiate the activity of VLPs as vehicles for generating a cell-mediated immune response (CMI), we determined whether this activity was retained by the chimeric VLPs. It has been shown previously that infectious virions, L1 VLPs and L1/L2 VLPs, can agglutinate mouse erythrocytes and that this hemagglutination (HA) correlates with cell surface binding to nucleated cells (27). In contrast, denatured VLPs or virions do not have HA activity. Both the BPV and HPV16 chimeric VLPs agglutinated mouse erythrocytes in a similar fashion to L1/L2 VLPs, requiring 30–60 ng of VLPs to induce HA in our standard assay (27). These results confirm the assembly state of the chimeric VLPs and indicate that they interact normally with cell surfaces.

Because we wished to produce a vaccine candidate with prophylactic as well as therapeutic potential against HPV16 infection, the ability of the chimeric particles to induce neutralizing antibodies was tested. Chimeric VLP antisera were prepared in rabbits and virion antibody titers were compared with those of antisera generated against the parental L1/L2 VLPs in two assays. In a HA inhibition assay (28), which measures the subset of neutralizing antibodies that inhibit virion cell surface binding, the sera elicited by both the BPV and HPV chimeras, and their corresponding parental VLPs, completely inhibited HA to a dilution of 1:1,600 (Fig. 4, data shown for HPV VLPs).

Sera to the BL1/L2-16E7 chimera were also directly tested for their ability to neutralize BPV infection of mouse C127 cells, as measured by a reduction in the number of foci induced by a standard amount of BPV virions (5). The immune sera

FIG. 1. Coimmunoprecipitation of 16L2–16E7 or 16L2-CE2 with 16L1 when coexpressed in insect cells. (*A*) Purified VLPs consisting of 16L1 alone (lanes a), 16L1 and 16L2 (lanes b), or 16L1 and 16L2–16E7 (lanes c) were immunoprecipitated with anti-16L1 mAb V.5 and analyzed by Western blotting with anti-GST 16L2 (*Left*) or anti-trp-16E7 (*Right*). (*B*) Crude lysates containing 16L1 and 16L2 (lane a), 16L2-CE2 (lane b), or 16L1 and 16L2-CE2 (lane c) were immunoprecipitated with anti-16L1 mAb V.5 and analyzed by Western blotting with anti-GST 16L2.

FIG. 3. Cosedimentation of BL2–16E7 fusion protein with BL1 when coexpressed in Sf9 insect cells. Coexpressed BL1 and BL2–16E7 were purified on CsCl gradients and the proteins were separated on a 12–45% linear sucrose gradient. Gradient fractions (numbered at the top) were analyzed by Coomassie staining of SDS/PAGE for L1 (row A) and by Western blotting with GST-BPV1 L2 antiserum (row B), or trp-HPV16 E7 antiserum (row C).

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FIG. 4. Antibodies to chimeric VLPs inhibit VLP-mediated HA. A total of 60 ng of HPV16 L1/L2 VLPs were incubated for 1 h at ambient temperature with two-fold dilutions (1:400 to 1:3200, left to right) of prebleed serum or rabbit anti-VLP sera. Antisera were made either to native VLPs (N) or denatured VLPs (D). The samples were mixed with mouse erythrocytes at a final concentration of 0.5% (vol/vol) in 100 μl of PBS/0.1% BSA per well of a 96-well plate, incubated for 3 h at 4°C, and photographed.

generated by inoculation with baculovirus-derived chimeric VLPs were able to reduce the infectivity of the BPV by 50% at a dilution of at least 1:30,000 (a titer of 30,000), which was equivalent to the neutralizing titer of the control serum raised against BPVL1/L2 VLPs (data not shown). The preimmune sera did not inhibit focal transformation at a dilution of 1:30, the lowest dilution tested.

The ability of anti-16L1/L2–16E7 serum to inhibit HPV16 infection was examined also by using *in vitro*-generated HPV16 pseudotype virus (29). As with BPV, the sera to the HPV16 chimera and wild-type VLP had similar neutralizing activities; both were able to reduce the infectivity of the pseudotype HPV16 virus by 50% at a dilution of at least 1:10,000 (a titer of 10,000) (Fig. 5). Taken together, these results indicate that the incorporation of the L2-E7 fusion protein into VLPs does not affect the presentation of conformationally dependent immunodominant epitopes on L1.

The sera generated to intact and denatured chimeric VLPs were also screened for antibodies to HPV16 E7. No E7 antibodies were detected in the sera of rabbits inoculated with intact HPV16 or BPV chimeras as assayed by immunoblotting against a bacterially derived 6-His-E7 fusion protein. However, E7 antibodies were detected when the chimeras were denatured prior to inoculation (data not shown). These results suggest that E7 has an internal location in the chimeric particles and is not accessible for generating an antibody response unless the particles are disassembled.

Generation of an L2-E2 Chimera. To examine the potential for incorporating larger proteins into chimeric VLPs, an L2-E2 chimera was generated by fusing the full-length CRPV E2 (391 aa) to the C-terminus of HPV16 L2 (16L2-CE2), and the chimera was coexpressed with HPV16 L1. Expression of the chimera was verified on immunoblots by the detection of a protein product with the expected apparent molecular weight
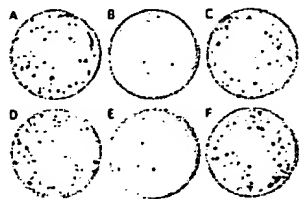
FIG. 5. Rabbit antiserum to 16L1/L2-16E7 VLPs neutralizes *in vitro* generated pseudotype HPV16 virions. HPV16L1/L2 recombinant SFV stock was used to infect BPHE-1 cells harboring the BPV1 genome (29). Cell lysates containing the resulting pseudotype HPV16 virions were incubated with preimmune at a dilution of 1:50 (*A*) or immune serum to HPV16 L1/L2 VLPs at dilutions of 1:10,000 (*B*) or 1:30,000 (*C*), or preimmune at a dilution of 1:50 (*D*) or immune serum to HPV16 L1/L2-16E7 VLPs at dilutions of 1:10,000 (*E*) or 1:30,000 (*F*), and placed onto monolayers of C127 cells. After 3 weeks, the cells were stained with 0.5% (wt/vol) methylene blue/0.25% (wt/vol) carbol fuschin in methanol, and the number of foci was scored (45).

of ≈120 kDa that reacted with HPV16 L2 antibodies (Fig. 1*B*). As with the E7 chimeras, the L2-E2 chimera coassembled with L1 into VLPs, as shown by TEM (Fig. 2*C*), cosedimented with L1 in a sucrose gradient (data not shown), and coimmunoprecipitated with L1 antisera (Fig. 1*B*). The HA activity of the L2-E2 chimera was also similar to the L1/L2 wild type, requiring 60 ng of protein in a standard assay.

Antitumor Responses Elicited by E7 Chimeric VLPs. To examine whether the chimeric VLPs could generate protective CMI, C57BL/6 (B6) mice immunized with either wild-type or HPV16 E7 chimeric VLPs were tested for protection against subsequent tumor challenge by using a B6 mouse tumor cell line, TC-1, that was developed to examine E7 specific antitumor activity (16). TC-1 expresses E7, but not L1 or L2. Protection from challenge with TC-1 was demonstrated previously when HPV16 E7 was expressed via a recombinant vaccinia virus and targeted to lysosomes via a lysosomal-associated membrane protein (LAMP) sorting signal to increase CD4$^+$ T helper responses (16). When injected without adjuvant, the 16L1/L2-16E7 chimeric VLPs were able to induce protection from tumor challenge (Table 1 and Fig. 6*A*). In contrast, aggressively growing tumors were observed in most animals vaccinated with the wild-type 16L1/L2 VLPs. Therefore, the results indicate that chimeric papillomavirus VLPs can induce antitumor immunity that is specific for the inserted polypeptide. Complete protection was obtained after a single vaccination with 10 μg of the E7 chimeric VLPs (Table 1).

To examine the mechanism of protection from tumor challenge elicited by the E7 chimeric VLPs, we tested the ability of the chimeric VLPs to protect B6-derived mice with germ-line defects in β_2-microglobulin, perforin, or MHC class II expression (23–25). Good protection was observed in the MHC class II-deficient mice, which lack mature CD4$^+$ T cells (Table 1 and Fig. 6*B*). In contrast, no significant protection was observed in the β_2-microglobulin knockouts ($P = 0.0001$ by using the vaccinated B6 mice as the reference), implying that protection requires the activity of MHC class I-restricted cytotoxic lymphocytes (CTLs). Protection is, at least in part, perforin-mediated because the perforin knockout mice were also not protected ($P = 0.005$ compared with the vaccinated B6 mice). NK cell-depleted B6 mice (NK depletion; data not shown) were protected from tumor challenge (Fig. 6*B*), supporting the idea that CD8$^+$ CTLs were the prime mediators of protection. It is very unlikely that antibody responses play a critical role in protection from tumor challenge. The unprotected animals inoculated with parental VLPs and the protected animals inoculated with the chimeric VLPs generated similar titers of

Table 1. HPV16 L1/L2-16E7 VLPs protect mice against challenge from TC-1 tumor cells

Mouse strain	Immunogen	Exp. 1	Exp. 2	*P* value
B6	PBS	3/4	4/5	Ref
B6	L1/L2	4/5	5/5	0.5
B6	L1/L2-E7	0/5	1/5	0.005
B6	L1/L2-E7*	ND	0/5	0.05
β_2m KO	L1/L2-E7	5/5	5/5	1.0
Perforin KO	L1/L2-E7	3/5	5/5	1.0
Class II KO	L1/L2-E7	1/5	1/5	0.02
NK depleted	L1/L2-E7	1/5	0/5	0.005

The results for experiments 1 and 2 are reported as the number of tumor-positive animals per total number of animals challenged. The *P* values were determined for the comparison of tumor take in the VLP-immunized mice to that of PBS-vaccinated controls and was calculated using the sum of the results of the two experiments. B6, C57Bl/6; β_2m KO, β_2-microglobulin knockout; Class II, MHC class II knockout; Perforin KO, perforin knockout; NK depleted, C57Bl/6 depleted of natural killer cells; L1/L2, L1/L2 virus-like particles; L1/L2-E7, L1/L2-E7 chimeric virus-like particles.
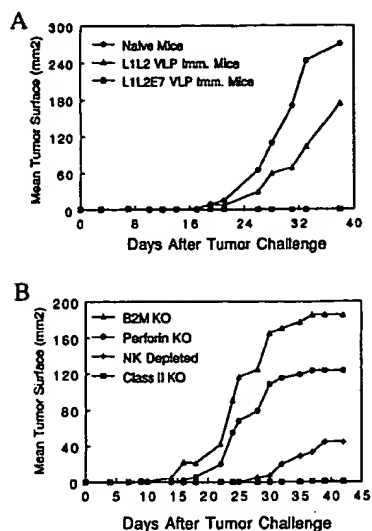*Vaccinated only once with 10 μg VLPs in PBS; ND, not determined.

FIG. 6. Growth of TC-1 tumor cells in VLP-vaccinated mice. The mean tumor area in the days following tumor challenge is shown for experiment 1 of Table 1. (*A*) Results from C57BL/6 mice immunized with L1/L2 or L1/L2-E7 VLPs are compared with results from mice injected with PBS (naive). (*B*) Results after L1/L2-E7 vaccination of C57BL/6 derived β_2-microglobulin knockout (B2M KO), perforin knockout, MHC class II knockout, or NK cell-depleted C57BL/6 mice are shown.

VLP antibodies (mean titer of 100,000 for both L1/L2 and L1/L2-E7 injected mice); none of the animals produced antibodies to E7.

DISCUSSION

This study used the L2 minor capsid protein for the incorporation of heterologous proteins into papillomavirus VLPs. Our generation of E7 and E2 chimeric papillomavirus VLPs demonstrates that entire proteins at least as large as the 391-aa CRPV E2 can be incorporated into papillomavirus VLPs as fusions with L2. Because E7 and E2 are not structurally related, these findings raise the possibility that papillomavirus VLPs could generally act as vaccine vehicles for delivery of protein antigens in their entirety, thus incorporating all of a protein's potentially immunogenic epitopes.

Chimeric VLPs incorporating foreign antigens have been generated previously by using capsid proteins from other viruses, including parvovirus, HIV-1, and hepatitis virus (30–32), but they have not been shown to generate antitumor immunity. Furthermore, the foreign peptides have usually been inserted into the major capsid protein, whose ability to self-assemble limited the size of the insert to relatively small peptides. For instance, a maximum of 21 aa from HPV16 E7 was inserted into hepatitis B core antigen particles (31), and similar size constraints have been noted for HPV L1 (33). The insertion of a larger polypeptide, the 147-aa hen egg white lysozyme protein, into parvovirus capsids was presumably accomplished because it was fused to VP1, the parvovirus minor capsid protein (32).

Our results strongly suggest that incorporation of the extra polypeptides did not perturb the outer structure of the papillomavirus VLP. The morphology of the parental L1/L2 and chimeric VLPs were indistinguishable in electron micrographs, and the chimeric and parental particles possessed indistinguishable HA activities. Most importantly, the chimeric VLPs also had wild-type activity in inducing neutralizing antibodies. It was critical to demonstrate the retention of this activity because one of the primary goals of the study was to produce a candidate HPV vaccine that has the potential of generating

effective immunoprophylactic as well as immunotherapeutic responses in human trials.

In outbred human populations, chimeric papillomavirus VLP-based vaccines could have a potential advantage over peptide-based strategies because protection would not be HLA allele restricted. Also, peptide vaccination can, in some instances, unexpectedly lead to enhanced tumor growth through the induction of specific T cell tolerance (34, 35). Chimeric VLPs also have a theoretical advantage over "naked" DNA or recombinant virus mediated strategies for inducing cytotoxic T cell responses in that there is no possibility for stable expression of the target protein. This characteristic is particularly advantageous in the case where oncoproteins, such as E7, are the immunogen because oncogenes are unacceptable vaccine candidates in many situations.

MHC class I-restricted responses by CTLs are the principal effectors of protective immunity to noncytopathic viruses and tumors (36, 37). It is highly likely that the antitumor response to the E7 chimeric VLPs measured here is mediated by CD8$^+$ CTLs because protection was not seen in the β_2-microglobulin or perforin knockout mice but was observed in the MHC class II-deficient mice or after NK cell depletion. It is conceivable that vaccination with VLPs could induce MHC class I-restricted CTLs by two mechanisms. Both L1 and L1/L2 VLPs mimic authentic virions in the avid binding to the papillomavirus cell surface receptor and subsequent internalization (14, 15). Therefore, they may also mimic authentic virus in the escape of endocytic vesicles into the cytoplasm where uncoating must occur to initiate the viral replication cycle. This result would permit presentation of the viral capsid proteins by the normal endogenous route. Alternatively, the VLPs may be presented via an exogenous pathway that preferentially processes particulate antigen through macrophages, or other phagocytic cells, such as subsets of dendritic cells (38, 39).

At the present time, it is unclear what physical aspects of VLPs lead to their effective induction of an antitumor response in the absence of adjuvant or MHC class II-restricted functions. It is possible that multiple features, including their ability to bind to cell surfaces, their particulate and repetitive structure (40, 41), and their presumed ability to escape endocytic vesicles into the cytoplasm, play a role in their ability to elicit CMI that appears to be T helper-independent. Our results with the chimeric VLPs are in contrast to those obtained after vaccination with an E7 expressing vaccinia virus. With the vaccinia vector, protection against TC-1 challenge was not observed unless E7 was targeted to lysosomes via a LAMP sorting signal, with the resulting enhancement of CD4$^+$ T helper responses (16). We have found that class II-restricted functions are not required for efficacy of the VLP-based vaccine in this tumor model. Therefore, VLPs may be particularly advantageous for generating CTL responses in situations where chronic exposure to the target tumor antigen(s), in the absence of costimulation, has resulted in T helper tolerance or exhaustion (42).

Practically, the ability to induce CMI in the absence of adjuvant reduces the likelihood that vaccine administration will produce adverse inflammatory side effects. In addition, the ability to induce potent antigen-specific CMI in the absence of adjuvant suggests that the potential for inducing undesirable autoimmune reactions might be less by using chimeric VLPs relative to strategies employing potent nonspecific immune modulators. These are especially important considerations in the development of a combined prophylactic/therapeutic genital HPV vaccine, because the target population for the vaccine is predominantly healthy adolescents, relatively few of which are destined to develop HPV-induced pathologies.

Although protection from experimental tumor challenge in mice was elicited by a single 10 μg VLP inoculation, multiple boosts will probably be required to induce optimal immunity for eradication of established disease such as micrometastases following debulking of a local pelvic tumor by surgery or irradiation.

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An attractive feature of a papillomavirus VLP-based therapeutic vaccine strategy is the potential for multiple boosts without the possibility that antibody responses to previous inoculations will influence antigen presentation. Many papillomavirus types have been identified (more than 70 in humans) and their virions are remarkably type specific in their ability to generate neutralizing antibodies (43, 44). As a consequence, antibodies generated by one papillomavirus VLP type do not prevent normal cell surface interactions by other VLP types (28). Therefore, multiple papillomavirus VLP types incorporating the same chimeric protein could be used to boost the CMI to the inserted protein, whereas the immune system of the host remains naive to the vehicle in each successive round of inoculation. Our incorporation of HPV16 E7 into both HPV16 and BPV1 VLPs demonstrates the technical feasibility of this approach. Its feasibility is further supported by the fact that no antibody response to the E7 insert was detected after chimeric VLP vaccination, presumably because the inserted polypeptide projects into the interior of the empty capsid. Several types of CRPV E2 chimeric VLPs, including the HPV16L1/L2-CE2 chimera reported here, have been generated for use in therapeutic vaccine trials to examine regression of established CRPV papillomas after multiple rounds of inoculation with different chimeric VLP types (our unpublished results).

Several human clinical trials to examine the safety and immunogenicity of HPV L1 or L1/L2 VLP-based vaccines will almost certainly be conducted within the next few years (12). The results of this study suggest that human testing of E7 and/or E2 chimeric VLPs should also be considered. If the human safety profiles and serologic responses to the chimeric and parental particles are equivalent and evidence for CMI responses to the inserted protein are obtained, then the chimeric VLPs' greater potential for inducing therapeutic responses against established HPV-induced lesions would make them an attractive candidate in future efficacy trials of genital HPV VLP-based vaccines.

1. World Health Organization (1995) *Human Papillomaviruses*, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans (World Health Organization, Lyons, France), Vol. 64.
2. Bosch, F. X., Manos, M. M., Munoz, N., Sherman, M., Jansen, A. M., Peto, J., Schiffman, M. H., Moreno, V., Kurman, R. & Shah, K. V. (1995) *J. Natl. Cancer Inst.* 87, 796–802.
3. Pisani, P., Parkin, D. M. & Ferlay, J. (1993) *Int. J. Cancer* 55, 891–903.
4. Schiller, J. T. & Roden, R. B. S. (1996) in *Papillomavirus-Like Particles: Basic and Applied Studies*, ed. Lacey, C. (Leeds Medical Information, Leeds, U.K.), pp. 101–112.
5. Kirnbauer, R., Booy, F., Cheng, N., Lowy, D. R. & Schiller, J. T. (1992) *Proc. Natl. Acad. Sci. USA* 89, 12180–12184.
6. Kirnbauer, R., Taub, J., Greenstone, H., Roden, R. B. S., Durst, M., Gissmann, L., Lowy, D. R. & Schiller, J. T. (1993) *J. Virol.* 67, 6929–6936.
7. Roden, R. B. S., Weissinger, E. M., Henderson, D. W., Booy, F., Kirnbauer, R., Mushinski, J. F., Lowy, D. R. & Schiller, J. T. (1994) *J. Virol.* 68, 7570–7574.
8. Lowy, D. R., Kirnbauer, R. & Schiller, J. T. (1994) *Proc. Natl. Acad. Sci. USA* 91, 2436–2440.
9. Breitburd, F., Kirnbauer, R., Hubbert, N. L., Nonnenmacher, B., Trin-Dinh-Desmarquet, C., Orth, G., Schiller, J. T. & Lowy, D. R. (1995) *J. Virol.* 69, 3959–3963.
10. Kirnbauer, R., Chandrachud, L., O'Neil, B., Wagner, E., Grindlay, G., Armstrong, A., McGarvie, G., Schiller, J., Lowy, D. & Campo, M. (1996) *Virology* 219, 37–44.
11. Suzich, J. A., Ghim, S., Palmer-Hill, F. J., White, W. I., Tamura, J. K., Bell, J., Newsome, J. A., Jenson, A. B. & Schlegel, R. (1995) *Proc. Natl. Acad. Sci. USA* 92, 11553–11557.
12. Steller, M. A. & Schiller, J. T. (1996) *J. Natl. Cancer Inst. Monogr.* 21, 145–148.
13. Stoler, M. H., Rhodes, M. H., Witbeck, A., Wolinsky, S. M., Chow, L. T. & Broker, T. R. (1992) *Hum. Pathol.* 23, 117–128.
14. Muller, M., Gissmann, L., Cristiano, R. J., Sun, X. Y., Frazer, I. H., Jenson, A. B., Alonso, A., Zentgraf, H. & Zhou, J. (1995) *J. Virol.* 69, 948–954.
15. Roden, R. B. S., Kirnbauer, R., Jenson, A. B., Lowy, D. R. & Schiller, J. T. (1994) *J. Virol.* 68, 7260–7266.
16. Lin, K.-Y., Guarnieri, F. G., Staveley-O'Carroll, K. F., Levitsky, H. I., August, J. T., Pardoll, D. M. & Wu, T.-C. (1996) *Cancer Res.* 56, 21–26.
17. Feltkamp, M. C. W., Smits, H. L., Vierboom, M. P. M., Minnaar, R. P., deJongh, B. M., Drijfhout, J. W., Ter Schegget, J., Melief, C. J. M. & Kast, W. M. (1993) *Eur. J. Immunol.* 23, 2242–2249.
18. Feltkamp, M. C. W., Vreugdenhil, G. R., Vierboom, M. P. M., Ras, E., Van der Burg, S. H., Ter Schegget, J., Melief, C. J. M. & Kast, W. M. (1995) *Eur. J. Immunol.* 25, 2638–2642.
19. Innis, M. A., Gelfand, D. H., Sninsky, J. J. & White, T. J. (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic, New York).
20. Barbosa, M. S., Vass, W. C., Lowy, D. R. & Schiller, J. T. (1991) *J. Virol.* 65, 292–298.
21. Christensen, N. D., Dillner, J., Eklund, C., Carter, J. J., Wipf, G. C., Reed, C. A., Cladel, N. M. & Galloway, D. A. (1996) *Virology* 223, 174–184.
22. Montross, L., Watkins, S., Moreland, R. B., Mamon, H., Caspar, D. L. & Garcea, R. L. (1991) *J. Virol.* 65, 4991–4998.
23. Kagi, D., Ledermann, B., Burki, K., Seiler, P., Odermatt, B., Podack, E. R. & Zinkernagel, R. M. (1994) *Nature (London)* 369, 31–37.
24. Grusby, M. J., Johnson, R. S., Papaioannou, V. E. & Glimcher, L. H. (1991) *Science* 253, 1417–1420.
25. Zijlstra, M., Li, E., Sajjadi, F., Subramani, S. & Jaenish, R. (1989) *Nature (London)* 342, 435–438.
26. Karlhofer, F. M. & Yokoyama, W. M. (1991) *J. Immunol.* 149, 3662–3673.
27. Roden, R. B. S., Hubbert, N. L., Kirnbauer, R., Breitburd, F., Lowy, D. R. & Schiller, J. T. (1995) *J. Virol.* 69, 5147–5151.
28. Roden, R. B. S., Hubbert, N. L., Kirnbauer, R., Christensen, N. D., Lowy, D. R. & Schiller, J. T. (1996) *J. Virol.* 70, 3298–3301.
29. Roden, R. B. S., Greenstone, H. L., Kirnbauer, R., Booy, F. P., Jessie, J., Lowy, D. R. & Schiller, J. T. (1996) *J. Virol.* 70, 5875–5883.
30. Wagner, R., Deml, L., Schirmbeck, R., Niedrig, M., Reimann, J. & Wolf, H. (1996) *Virology* 220, 128–140.
31. Tindle, R. W., Herd, K. & Londono, P. (1994) *Virology* 200, 547–557.
32. Miyamura, K., Kajigaya, S., Monoeda, M., Smith-Gill, S. J. & Young, N. S. (1994) *Proc. Natl. Acad. Sci. USA* 91, 8507–8511.
33. Muller, M., Zhou, J., Reed, T. D., Rittmuller, C., Burger, A., Gabelsberger, J., Braspenning, J. & Gissmann, L. (1997) *Virology* 234, 93–111.
34. Toes, R. E. M., Offringa, R., Blom, R. J. J., Melief, C. J. M. & Kast, W. M. (1996) *Proc. Natl. Acad. Sci. USA* 93, 7855–7860.
35. Toes, R. E. M., Blom, R. J. J., Offringa, R., Kast, W. M. & Melief, C. J. M. (1996) *J. Immunol.* 156, 3911–3918.
36. Zinkernagel, R. M., Bachmann, M. F., Kundig, T. M., Oehen, S., Pirchet, H. & Hengartner, H. (1996) *Annu. Rev. Immunol.* 14, 333–367.
37. Kast, W. M. & Melief, C. J. M. (1991) *Immunol. Lett.* 30, 229–232.
38. Bachmann, M. F., Lutz, M. B., Layton, G. T., Harris, S. J., Fehr, T., Rescigno, M. & Ricciardi-Castagnoli, P. (1996) *Eur. J. Immunol.* 26, 2595–2600.
39. Kovacsovics-Bankowski, M., Clark, K., Benacerraf, B. & Rock, K. L. (1993) *Proc. Natl. Acad. Sci. USA* 90, 4942–4946.
40. Baker, T. S., Newcomb, W. W., Olson, N. H., Cowsert, L. M., Olson, C. & Brown, J. C. (1991) *Biophys. J.* 60, 1445–1456.
41. Bachmann, M. F. & Zinkernagel, R. M. (1996) *Immunol. Today* 17, 553–558.
42. Matzinger, P. (1994) *Annu. Rev. Immonol.* 12, 991–1045.
43. Roden, R. B. S., Greenstone, H. L., Kirnbauer, R., Booy, F. P., Jessie, J., Lowy, D. R. & Schiller, J. T. (1996) *J. Virol.* 70, 5875–5883.
44. Christensen, N. D., Kirnbauer, R., Schiller, J. T., Ghim, S. J., Schlegel, R., Jenson, A. B. & Kreider, J. W. (1994) *Virology* 205, 329–335.
45. Dvoretzky, I., Shober, R., Chattopadhyay, S. K. & Lowy, D. R. (1980) *Virology* 103, 369–375.

REVIEW

Papillomavirus-Like Particles

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Overview

The discovery that the major capsid protein of papillomaviruses assembles into virus-like particles (VLPs), in the absence of other viral proteins, has led to significant advances in the study of the biology of these DNA tumour viruses. VLPs present the conformational epitopes required for generating high titre neutralising antibodies but are devoid of the potentially oncogenic viral genome. Therefore, the VLPs are attractive candidates for a vaccine to prevent genital HPV infection, a condition strongly associated with cervical and other anogenital carcinomas. HPV VLPs have also been exploited as an antigen in enzyme-linked immunosorbant assays (ELISAs) to detect anti-virion immune responses in human sera. VLP-based ELISAs for genital types have sufficient sensitivity and specificity to make them widely applicable to studies of the natural history of HPV infection and its association with neoplastic disease.

Virion assembly and structure

Papillomavirus (PV) virions are 55-60 nm diameter non-enveloped icosahedral structures containing an approximately 8Kb double stranded covalently closed circular DNA genome packaged as a nucleohistone complex (1). The capsid surrounding the nucleohistone core is composed of the major and minor capsid proteins, designated L1 and L2 respectively (2-4). The capsid has T=7d icosahedral symmetry and is composed of 72 pentameric capsomers (5). Computer generated reconstructions from cryo-electron micrographs indicate that the capsomers at the 12 vertices of the virion contact five other capsomers (pentavalent), whereas the remaining sixty capsomers contact six other capsomers (hexavalent)

(6). PV virions superficially resemble SV40 and polyoma virions in this regard, but in contrast to SV40 and polyoma (7,8), no high resolution X-ray crystallographic structure has been determined for a papillomavirus.

Because of the constraints imposed by the currently available systems for generating PV virions (9), the location, structure and function of the L1 and L2 proteins within the capsid were unclear from studies of virions. The analysis of VLPs has provided an insight into the role of L1 and L2 in capsid formation. L1 alone from all papillomavirus types tested to date (Table 1) assembles into capsid-like structures when expressed at sufficiently high levels in eukaryotic cells, indicating that structural elements of most, if not all, of the capsomers are composed of L1 (10). An early study reported that HPV 16 L1 was unable to self-assemble in the absence of L2 (11). However, the L1 of the reference strain used in these experiments was later shown to be an assembly-deficient mutant (12). Most HPV 16 L1 proteins, and all that efficiently self-assemble, contain an aspartate at position 202 rather than the histidine found in the L1 of the reference strain (13, and R. Kimbauer, unpublished results). VLPs have been generated using several eukaryotic expression systems including baculovirus, vaccinia virus, Semliki Forest virus (14) and yeast (Table 1). In contrast, L1 forms insoluble aggregates when produced in bacteria, suggesting that virion formation may be dependent on factors present only in eukaryotic cells (15,16). Whether the cellular factors are simply involved in the proper folding of L1 monomers or also in later steps of the assembly process remains to be determined.

When L2 is co-expressed with L1 in eukaryotic cells, it is stably incorporated into the capsids at a ratio of

Table 1

PV type	Expression system	Reference
BPV1	Baculovirus	(10)
	Vaccinia virus	(25)
	Semliki Forest Virus	Roden, unpublished
BPV4	Baculovirus	Kimbauer, unpublished
COPV	Baculovirus	Ghim, unpublished
CRPV	Baculovirus	(12)
RhPV	Baculovirus	Kimbauer, unpublished
HPV 1	Vaccinia virus	(19)
HPV 6	Baculovirus	(12, 27)
	Yeast	(20, 33)
HPV 11	Baculovirus	(12, 27, 48, 51)
HPV 16	Baculovirus	(12, 48, 68, 69)
	Vaccinia virus	(70)*
	Semliki Forest Virus	Roden, unpublished
	Yeast	(33)
HPV 18	Baculovirus	(31, 48)
HPV 31	Baculovirus	(31)
HPV 33	Baculovirus	(18)
HPV 45	Baculovirus	Kimbauer, unpublished

*Particles approximately 40Å in diameter.

approximately one L2 molecule per 30 L1 molecules (12,17-20). Both L1 alone and L1/L2 VLPs have a capsid structure that was morphologically indistinguishable from virions in computer reconstructions of cryo-electron micrographs at a resolution of 35 Å (21). Therefore, the position of L2 within the capsid remains to be determined. HPV 16 L2 enhanced capsid formation four-fold in insect cells whereas HPV 1 L2 enhanced capsid formation 100-fold in a vaccinia expression system (12,19). This discrepancy may be due to differences in the relative levels of L1 expression in the two systems. In all VLP preparations, partially assembled capsids and rod-like structures are also detected (18). Similar rod-like particles have also reported in preparations of authentic virions (22). Only a small portion of the L2 protein may be exposed on the surface of BPV virions or VLPs, since neutralising epitopes have been detected in only a relatively short N-terminal segment of the protein (23). The N-terminus of L2 has been reported to bind non-specifically to DNA *in vitro*, suggesting that it may be involved in an interaction with the viral genome in the virion (24).

The ability to produce infectious PV virions *in vitro* would open a number of aspects of PV biology to investigation. In an attempt to promote encapsidation of the PV genome DNA using the VLP technology, the BPV capsid proteins have been produced, using vaccinia (25)

and Semliki-Forest virus-based expression vectors (our unpublished results), in cells that maintain the BPV genome as a high copy autonomous replicon. Low levels of infectious BPV are produced under these conditions, as demonstrated by the cell-free transfer of expression of early PV RNA (25) and focus formation in C127 cells (our unpublished results). In these studies, expression of both L1 and L2 was necessary for production of infectious virus. Capsids composed of L1 alone did not contain papillomavirus DNA, suggesting that L2 has a specific function in genome encapsidation (25).

VLPs are likely to be important reagents in future studies of capsid structure. Unlike virions, the VLPs can easily be generated in quantities sufficient for crystallisation which may lead to a high resolution structural model for PV virions based on X-ray diffraction analysis. Since VLPs are produced in systems amenable to genetic manipulation, a number of virus-related processes can now be explored by mutagenesis of the capsid proteins, including virion assembly, the nature of the neutralising epitopes on the virion surface, and the interaction of the capsid with its cell surface receptor.

Early events in papillomavirus infection.

VLPs have been exploited in studies of the early events in PV infection. The presence of VLPs composed of BPV L1 or L1 plus L2 inhibited infection of mouse fibroblasts by BPV virions as assessed by focal transformation (26). This suggests that VLPs have affinity for the same cell surface receptor(s) as virions and that L2 is not required for this interaction. HPV 11 or HPV 16 VLPs also inhibited BPV infection, implying that these PVs share a common cell surface receptor or a downstream processing event during infection. Supporting the former possibility is the observation that the binding of BPV virions to cell surfaces (and the uptake of HPV 6b, 11 and 16 VLPs) is inhibited in the presence of heterologous VLPs (26,27).

HPV 11 and HPV 33 VLPs, and labelled BPV virions, bind a variety of cell lines of epithelial, mesenchymal and neuronal origin derived from a broad range of species, suggesting that the cell surface receptor for PVs is widely expressed and evolutionarily conserved (26-28). The tropism of many viruses is determined by the species or tissue distribution of their receptors. It is perhaps surprising, in light of the strict host range of the PVs, that this specificity appears to be determined by some post-binding event in the infectious cycle (29). However, the above analyses do not rule out the possibility that, in addition to a widely distributed primary receptor, a more specific secondary receptor may be involved in virus uptake.

It has been estimated that there are approximately 2.6 x 10⁴ cell surface HPV 33 VLP receptors per HeLa cell that

bind with a dissociation constant of 84 pM (28). Binding f VLPs with the cell surface receptor is decreased at high salt concentrations and abolished above pH 7.8, consistent with a polar interaction (28). Dissociated capsomers, produced from VLPs by divalent cation chelation under reducing conditions, neither bind to cell surfaces nor compete with VLP binding to cells, implying that inter-capsomer contacts are required for binding the receptor (28). Trypsin treatment of cell surfaces prior to addition of particles inhibited their binding to the cells (27,28). Although the virus receptor has not been identified, this suggests that it has a protein component. As yet there is no evidence for the involvement of carbohydrates in virion-receptor interaction (28).

BPV virions agglutinate mouse erythrocytes, but not those of other species including rat and human (30). VLPs composed of BPV L1 alone or L1 plus L2 and VLPs derived from a number of HPV types also agglutinate erythrocytes with the same species specificity as BPV virions (31). Therefore, mouse erythrocytes but not those from other species present a PV cell surface receptor. It is unclear whether the receptor on mouse erythrocytes is the same as that on nucleated cells, but the erythrocyte receptor is also cleaved by trypsin and antibodies that block PV binding to nucleated cells also inhibit haemagglutination (31). Perhaps a comparison of membrane proteins derived from mouse and rat erythrocytes with those from nucleated cells will aid in the identification of the PV cell surface receptor. More practically, the integrity of VLP preparations can be monitored by determining the amount of the preparation required for haemagglutination, since dissociated VLPs do not haemagglutinate. Also, antibody induced inhibition of haemagglutination presumably occurs by preventing binding of the VLPs to the surface of the erythrocyte. Therefore, haemagglutination inhibition can be used as a surrogate for virus neutralisation.

VLP-based vaccines

For several reasons, VLPs are currently the most attractive candidates for developing a prophylactic vaccine against genital HPV infection (32). First, they are likely to be safe as, unlike virions, they lack the potentially oncogenic viral genomes. In addition, VLPs can be generated in, and easily purified from insect cells or yeast (10,33), two cell types that have generated products that have been approved for use in human vaccine trials.

Neutralising antibody titres generally correlate well with protection in prophylactic viral vaccines (34), and PV VLPs present the conformational virion surface epitopes required for generating high titre neutralising antibodies. In neutralisation assays, the titres of sera generated in rabbits immunised with recombinant BPV and HPV 11 VLPs are similar to those generated against the corresponding

authentic virions (10,35). Most of the neutralising epitopes are conformational, since the titres of neutralising antibodies induced by denatured or disassembled VLPs, or capsid protein produced in bacteria, were at least 3 orders of magnitude lower than the titres induced by assembled VLPs (10,15). L1 alone and L1/L2 VLPs produced similar neutralising titres, indicating that L1 has the immunodominant neutralising epitopes (36). Many of the above observations have more recently been extended to HPV 11 VLPs using a mouse xenograft infection model (35,37-39). Immunisation with VLPs induced a strong antibody and T-cell proliferative response even when the VLPs were injected in the absence of adjuvant (H. Greenstone, unpublished results), suggesting that their polyvalent and particulate nature may facilitate presentation to the immune system.

Studies in animal models have demonstrated that immunisation with VLPs leads to type specific protection against experimental PV infection. Insect cell-derived CRPV L1 or L1/L2 VLPs, but not BPV VLPs, protected domestic rabbits from experimental challenge of the flank with high dose CRPV virus (5 x 10^{10} virions) (40). Both Freund's and Alum (which is approved for human use) proved effective as adjuvants. Passive transfer of immunoglobulins from rabbits immunised with CRPV VLPs protected naive rabbits against subsequent experimental CRPV challenge, indicating that protection was antibody mediated (40).

In an experimental mucosal model, BPV 4 L1 or L1/L2 VLPs administered in Alum protected calves from experimental BPV 4 challenge of the soft palate (R. Kimbauer and S. Campo, unpublished results). In a field trial of oral mucosal immunity, a high degree of protection from natural transmission of COPV was demonstrated in beagle dogs after intradermal injection of formalin-inactivated COPV papilloma extract (41). Although these studies did not involve VLPs, the assumption is that protection was due to the generation of neutralising virion antibodies. If this assumption is correct, these results provide important support for the feasibility of a VLP-based prophylactic vaccine against genital HPVs since they demonstrate protection of a mucosal site from natural transmission of a PV after simple systemic inoculation. The results of field trials using COPV VLPs as the immunogen are eagerly awaited.

Based on the encouraging results of the antigenicity and immunogenicity studies of PV VLPs in the animal studies described above, it is likely that human trials of a VLP-based prophylactic vaccine against genital HPVs will be undertaken in the near future. Several decisions concerning the components, protocol, and evaluation of the candidate vaccine will need to be made prior to the initiation of

human trials. The patients could be inoculated with either purified VLP protein (12,33,42) or with live recombinant vaccinia virus expressing the PV late proteins (11,19). Although the use of live recombinant vaccinia virus may offer some advantages in the stimulation of the immune system, these are probably outweighed by the significant risks associated with vaccinia virus infection, especially for immunodeficient patients (43,44). The number of genital HPV types that should be targeted is unclear as world-wide approximately 50% of cervical cancers contain HPV 16 but 80% contain HPV 16, 18, 31 or 45 (45). Protection against infection by at least these four types 'would be most desirable. Also, HPV 6 and HPV 11-induced genital warts are very common and often cause significant morbidity, so they too might be considered as vaccine targets (35,46,47).

To make an informed decision of the number of VLP types to include in a broad spectrum HPV vaccine, it will be important to evaluate the likelihood that protection will be type specific. Based upon the analysis of animal types (40), it is unlikely that VLPs of divergent HPV types, such as HPV 16 and 18, will induce cross-protection (48). However, it is possible that some degree of cross-protection may be generated for more closely related types such as HPV 16 and 31, HPV 18 and 45, or 6 and 11 (49). Efficient neutralisation of HPV 11 infection in the mouse xenograft model by antibodies to HPV 11 but not HPV 6 VLPs argues against this possibility (39). In the absence of neutralising assays for high risk types, surrogate assays, such as cross-reactivity in VLP-based ELISAs or haemagglutination inhibition assays, will have to be used to evaluate the potential for cross-protection (31,48,50).

The decision of whether to use L1 or L1/L2 VLPs must also be made. Most neutralising antibodies are generated against L1 epitopes, suggesting that L1 VLPs might be preferred as the simplest effective immunogen (10,51). However, the yield of VLPs are at least four-fold higher for L1/L2 than L1 alone and so production of the vaccine would be more cost effective, and the vaccine perhaps more stable, if L2 were included (12,19). Also, protective responses against bacterially-derived L2 have been documented in animal studies (52-56). Therefore, L1/L2 VLPs may prove more effective than L1 VLPs. However, at least one potentially negative complication of using L1/L2 VLPs can be envisioned. Since L2 has a sequence independent affinity for DNA (24,57), appreciably more cellular DNA may co-purify with L1/L2 VLPs than with L1 VLPs. Since cellular DNA is an undesirable contaminant of a vaccine preparation, it will be important to determine the DNA content of the two types of VLP preparations.

It is unlikely that an L1 or L1/L2 VLP vaccine will be effective in eliminating HPV induced lesions, since the late genes are not detectably expressed in progressed lesions or the replicating basal cells of benign lesions. Incorporation of early proteins into the VLPs might increase the therapeutic potential of a VLP-based vaccine. Since virions must escape endocytic vesicles to initiate infection, the VLPs may also be released into the cytoplasm, where the accompanying early viral peptide could be degraded, presented as part of an MHC-I complex, and thereby generate a cytotoxic T cell response. To explore this possibility, full-length HPV 16 L2-E7 chimeric proteins that are incorporated into L1 VLPs have been generated. There was no reduction in neutralising titres to BPV VLPs when the chimeric BPV L2s were incorporated in place of wild type L2. The cell-mediated immune responses to E7 are currently under investigation (H. Greenstone and J. Schiller, unpublished results).

VLP-based serological assay

Serologic detection of HPV infection has not become routine because previous assays based on early proteins or denatured capsid proteins lacked sufficient sensitivity or specificity. Prior to the generation of PV VLPs, no standard reagents were available to evaluate critically the immune response to conformational virion surface epitopes. Recently developed VLP-based ELISAs for genital and cutaneous HPVs have demonstrated the utility of anti-virion serum antibodies as markers of HPV infection. Of individuals reporting a history of foot warts, 89% were positive in the HPV 1 VLP-based assay as opposed to 53% of controls (58). In an HPV 11 VLP assay, 49% of condylomata acuminata patients had reactivities above that of the highest control (35). The results using a VLP-based ELISA for HPV 16 demonstrated that 59% of women with current HPV 16 infection, as measured by the presence of genital tract HPV 16 DNA, generate a detectable systemic IgG response, in contrast to 6% of women negative for genital HPV DNA (50).

The VLP ELISAs open up new areas of research in epidemiology and natural history studies. The ability of the ELISA to assess the spread of infection from easily collected and analysed blood samples could have public health implications. The importance of HPV infection in the aetiology of cervical and other cancers can now be quantitatively assessed without the concerns of sampling errors, especially in non-diseased controls, inherent in case/control studies using DNA-based HPV testing. Lastly the assays should greatly facilitate the design and evaluation of VLP-based vaccine trials.

Given the potential utility of the VLP ELISAs, it is important to critically evaluate these assays, particularly in comparison to PCR based HPV DNA testing, currently the most accepted method for evaluating HPV infection. In

most populations examined to date, at least 25% of women with genital HPV 16 or HPV 6/11 DNA were judged seronegative in the corresponding VLP ELISA (50,59,60). Assuming that detection of genital tract HPV DNA is an accurate indication of current genital HPV infection, there are at least four probable explanations for the lower sensitivity of the ELISAs. Some women may have such modest local genital infections that no systemic serologic response is triggered. This possibility is supported by the finding that women with HPV 16 DNA levels detectable by ViraPap were significantly more likely to be ELISA positive than were women whose HPV 16 DNA could only be detected by the more sensitive PCR assay (67% vs 33%) (50). Other women may not have been infected for a sufficient length of time to mount a detectable immune response. Supporting this explanation is the observation that women with persistent HPV 16 infection, as measured by repeated genital HPV 16 DNA positivity by PCR, were much more likely to be seropositive than women with transient HPV 16 DNA (83% vs 22%; L. Wideroff et al., manuscript submitted). Also seroconversions against HPV 16 VLPs have been detected many months after first detection of HPV 16 DNA (61).

The titres of virion antibodies may have waned in women with cervical cancer or other progressed lesions, which do not express the virion structural proteins or produce virions. Consistent with this possibility is the finding that seropositivity was significantly lower in cancer patients than in CIN III patients in both Colombia and Spain (62). Also several cases of transient VLP antibody responses, where HPV 6 or HPV 16 VLP antibody titres waned after clearance of viral DNA have been documented (61,63). Lastly, it is also probable that some women are constitutively unable to mount an antibody response to papillomavirus virions.

Unlike DNA based assays, VLP ELISAs appear to detect women previously, as well those currently, infected with genital HPVs. Several cases of seroconversion with persistence of VLP antibodies after loss of HPV DNA have been reported (61). In addition, seropositivity in women who are currently negative for genital tract HPV DNA is strongly correlated with their lifetime number of sexual partners (59, Dillner et al., and Wideroff et al., manuscripts submitted).

The VLP ELISAs appear to be reasonably type specific or at least type restricted. Women with genital HPV 6 or HPV 11 DNA were four fold more likely to react positively in the HPV 6 VLP assay compared to women with genital HPV DNA of other types (59). Assays based on HPV 6 and HPV 11 VLPs may partially discriminate between infection by these two closely related genotypes. Approximately half of the reactive sera reacted in only one of the two VLP ELISAs, while the other half reacted in both (60). Reactivity in the HPV 16 VLP ELISA was not associated with infection by low risk types HPV 6 or HPV 11 (50).

The degree to which the HPV 16 assay detects antibodies generated against other high risk HPVs is uncertain. Reactivity was associated with cervical DNA of other high risk types in the absence of HPV 16 DNA (OR 3.3), although less strongly than with HPV 16 DNA in the absence of other types (OR 9.0) (Wideroff et al., manuscript submitted). This might be due to cross-reactivity between different high risk types, or infection with other types might be a surrogate marker for previous exposure to HPV 16 infection. The question of type specificity should be resolved by the development and validation of ELISAs based on VLPs of other high risk HPV types.

The VLP ELISAs are currently being used to study the natural history of genital HPV infection. One controversial question being addressed is the prevalence of non-sexual transmission of high risk HPV infection. One study found that virgin teenage girls were uniformly seronegative in the HPV 16 VLP ELISA while a significant number of their sexually active counterparts were seropositive (64). Seroconversion was closely associated with conversion to HPV 16 (and in one case HPV 31) DNA positivity. In another study of younger children (age 1- 10 years), no specific reactivity to HPV 16 VLPs, as compared to BPV VLPs, was detected (our unpublished results). Since these results suggest a low prevalence of persistent non-sexually acquired infection, they support the idea that a prophylactic genital HPV vaccine could be effective if administered prior to the onset of sexual activity.

In other studies, the HPV 16 VLP ELISA has been employed to assess the risk associated with HPV infection for cervical and other cancers. In cross-sectional case-control studies of cervical cancer in Spain, Colombia and Sweden, seropositivity was strongly associated with disease (ORs 56.7, 3.8 and 9.5 respectively) (62,65). The differences in odds ratios primarily reflected differences in seroprevalence among controls rather than cases. The eight-fold higher incidence of cervical cancer in Colombian compared with Spanish women was mirrored in the higher incidence of seropositivity among Colombian controls. A prospective study of Finnish sera further demonstrated that the presence of HPV 16 virion antibodies was a predictor of subsequent development of cervical cancer (OR 13.2) (Lehtinen et al., manuscript submitted).

Seropositivity in the HPV 16 VLP ELISA was also associated with two other cancers previously associated with HPV 16 DNA: anal epidermoid (OR 30.4) (66), and vulvar carcinoma (OR 5.3) (A. Hildesheim et al., manuscript submitted). These results establish the utility of

the VLP ELISA in detecting associations between HPVs and specific cancers. Attention can now be turned to cancers for which the association with HPV infection is less clear. In this regard, it is interesting that seropositivity in the HPV 16 VLP assay was associated with oesophageal (OR 14.6) but not other head and neck cancers examined in a recent prospective study of Finnish cancer patients (Dillner *et al.*, in press).

The above results establishing the correlation between seropositivity in the HPV 16 ELISA and cancer in several populations raise the possibility that the assay could also have utility as a clinical screen for evaluating an individual's risk of developing cervical or other HPV-associated anogenital cancers. The finding that the ELISA preferentially detects women with persistent HPV 16 infection is also encouraging since patients with persistent infections are most likely at greater risk of progressing to cancer. However, in contrast to the Papanicolaou (Pap) test, the ELISA specifically identifies HPV infections rather than genital neoplasias. Most ELISA positive women, even in a high risk population such as Greenland, have no cervical cytological abnormalities (our unpublished results). This reflects the fact that most women (especially younger women) with genital tract HPV 16 have normal cervices (67) and the ability of the ELISA to detect past as well as current infection. In addition to having many false positives with respect to cervical disease, the current assay also has only moderate sensitivity in detecting women with HPV associated cervical neoplasias. Approximately one-quarter of women with CIN III and genital HPV 16 DNA were negative in the assay (50, 62).

It is unlikely that a VLP ELISA, even one based on multiple high risk types, could replace the Pap test as a primary screen for cervical cancer. However it is possible that the test may eventually prove useful as a clinical screen in some settings where women either have no access to, or refuse to undergo, Pap screening. For instance, persistent VLP ELISA positivity in older women who have not had a Pap test might prove to have sufficient predictive value in diagnosing high grade cervical neoplasia to warrant preferential follow up of these women. Clearly more studies are required before the clinical utility of genital HPV VLP ELISAs can be adequately assessed.

Acknowledgement

References

1. Pfister H. Papillomaviruses: general description, taxonomy, and classification. In: Salzman NP, Howley PM, eds. The Papovaviridae: Volume 2. The Papillomaviruses. New York: Plenum Press, 1987:1-38.

2. Favre M, Breitburd F, Croissant O, Orth G. Chromatin-like structures obtained after alkaline disruption of bovine and human papillomaviruses. *J Virol* 1977;21:1205-9.

3. Favre M. Structural polypeptides of rabbit, bovine, and human papillomaviruses. *J Virol* 1975;15:1239-47.

4. Doorbar J, Gallimore PH. Identification of proteins encoded by the L1 and L2 open reading frames of human papillomavirus 1a. *J Virol* 1987;61:2793-9.

5. Klug A, Finch JT. Structure of viruses of the papilloma-polyoma type: I. human wart viruses. *J Mol Biol* 1965;11:403-23.

6. Baker TS, Newcomb WW, Olson NH, Cowsert LM, Olson C, Brown JC. Structures of bovine and human papillomaviruses: analysis by cryoelectron microscopy and three-dimensional image reconstruction. *Biophys J* 1991;60:1445-56.

7. Stehle T, Yan Y, Benjamin T, Harrison S. Structure of murine polyomavirus complexed with an oligosaccharide receptor fragment. *Nature* 1994;369:160-3.

8. Liddington RC, Yan Y, Moulai J, Sahli R, Benjamin TL, Harrison SC. Structure of simian virus 40 at 3.8-A resolution. *Nature* 1991;354:278-84.

9. Hagensee M, Galloway D. Growing human papillomaviruses and virus-like particles in the laboratory. *Papillomavirus Rep* 1993;4:121-4.

10. Kirnbauer R, Booy F, Cheng N, Lowy DR, Schiller JT. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc Natl Acad Sci USA* 1992;89:12180-4.

11. Zhou J, Sun XY, Stenzel DJ, Frazer IH. Expression of vaccinia recombinant HPV 16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. *Virology* 1991;185:251-7.

12. Kirnbauer R, Taub J, Greenstone H, Roden R, Dürst M, Gissman L, *et al.* Efficient self-assembly of human papillomavirus type 16 L1 and L1-L2 into virus-like particles. *J Virol* 1993;67:6929-36.

13. Pushko P, Sasagawa T, Cuzick J, Crawford L. Sequence variation in the capsid protein genes of human papillomavirus type 16. *J Gen Virol* 1994;75:911-6

14. Liljestrom P, Garoff H. A new generation of animal cell expression vectors based on the Semliki Forest Virus replicon. *Biotechnology* 1991;9:1356-61.

15. Pilacinski WP, Glassman DL, Krzyzek PL, Robbins AK. Cloning and expression in Escherichia coli of the bovine papillomavirus L1 and L2 open reading frames. *Biotechnology* 1984;2:356-60

16. Lin Y-L, Borenstein LA, Selvakumar R, Ahmed R, Wettstein FO. Effective vaccination against papilloma development by immunization with L1 or L2 structural protein of cottontail rabbit papillomavirus. *Virology* 1992;187:612-9.

17. Xi SZ, Banks LM. Baculovirus expression of the human papillomavirus type 16 capsid proteins: detection of L1-L2 protein complexes. *J Gen Virol* 1991;72:2981-8.

18. Volpers C, Schirmacher P, Streeck RE, Sapp M. Assembly of the major and the minor capsid protein of human papillomavirus type 33 into virus-like particles and tubular structures in insect cells. *Virology* 1994;200:504-12.

19. Hagensee ME, Yaegashi N, Galloway DA. Self-assembly of human papillomavirus type 1 capsids by expression of the L1 protein alone or by coexpression of the L1 and L2 capsid proteins. *J Virol* 1993;67:315-22.

20. Hofmann K, Cook J, Joyce J, Brown DR, Schulz LD, George HA, *et al.* Sequence determination of human papillomavirus 6a and assembly of virus-like particles in Saccharomyces cerevisiae. *Virology* 1995;209:506-18.

21. Hagensee ME, Olson NH, Baker TS, Galloway DA. Three-dimensional structure of vaccinia virus-produced human papillomavirus type 1 capsids. *J Virol* 1994;68:4503-5.

22. Kiselev N, Klug A. The structure of viruses of the papilloma-polyoma type: V: tubular variants built of pentamers. *J Mol Biol* 1969;40:155-71.

23. Roden RB, Weissinger EM, Henderson DW, Booy F, Kirnbauer R, Mushinski F, *et al.* Neutralization of bovine papillomavirus by antibodies to L1 and L2 capsid proteins. *J Virol* 1994;68:7570-4.

24. Zhou J, Sun XY, Louis K, Frazer IH. Interaction of human papillomavirus type 16 capsid proteins with HPV DNA requires an intact L2 N-terminal sequence. *J Virol* 1994;68:619-25.

25. Zhou J, Stenzel DJ, Sun XY, Frazer IH. Synthesis and assembly of infectious bovine papillomavirus particles in vitro. *J Gen Virol* 1993;74:763-8.

26. Roden RB, Kirnbauer R, Jenson AB, Lowy DR, Schiller JT. Interaction of papillomaviruses with the cell surface. *J Virol* 1994;68:7260-6.

27. Muller M, Gissmann L, Cristiano RJ, Sun X-Y, Frazer IH, Jenson AB, *et al.* Papillomavirus capsid binding and uptake by cells from different tissues and species. *J Virol* 1995;69:948-54.

28. Volpers C, Unckell F, Schirmacher P, Streeck RE, Sapp M. Binding and internalization of human papillomavirus type 33 virus-like particles by eukaryotic cells. *J Virol* 1995;69:3258-64.

29. Koller L, Olson C. Attempted transmission of warts from man, cattle, and horses and of deer fibroma, to selected hosts. *J Invest Dermatol* 1972;58:366-8.

30. Favre M, Breitburd F, Croissant O, Orth G. Hemagglutinating activity of bovine papilloma virus. *Virology* 1974;60:572-8.

31. Roden RBS, Hubbert NL, Kirnbauer R, Breitburd F, Lowy DR, Schiller JT. Papillomavirus L1 capsids agglutinate mouse erythrocytes through a proteinacieous receptor. *J Virol* 1995;69:5147-51.

32. Muñoz N, Crawford L, Coursaget P. HPV vaccines and their potential use in the prevention and treatment of cervical neoplasia. *Papillomavirus Rep* 1995;6:54-5.

33. Sasagawa T, Pushko P, Steers G, *et al.* Synthesis and assembly of virus-like particles of human papillomaviruses type 6 and type 16 in fission yeast Schizosaccharomyces pombe. *Virology* 1995;206:126-35.

34. Robbins JB, Schneerson R, Szu SC. Hypothesis: serum IgG antibody is sufficient to confer protection against infectious diseases by inactivating the inoculum. *J Infect Dis* 1995;171:1387-98.

35. Rose RC, Reichman RC, Bonnez W. Human papillomavirus type 11 recombinant virus-like particles induce the formation of neutralizing antibodies and detect HPV-specific antibodies in human sera. *J Gen Virol* 1994;75:2075-9.

36. Lowy DR, Kirnbauer R, Schiller JT. Genital human papillomavirus infection. *Proc Natl Acad Sci USA* 1994;91:2436-40.

37. Christensen ND, Kreider JW. Antibody-mediated neutralization in vivo of infectious papillomaviruses. *J Virol* 1990;64:3151-6.

38. Christensen ND, Kreider JW, Cladel NM, Patrick SD, Welsh PA. Monoclonal antibody mediated neutralization of infectious human papillomavirus type 11. *J Virol* 1990;64:5678-81.

39. Christensen ND, Kirnbauer R, Schiller JT, Ghim S-J, Schlegel R, Jenson AB, Kreider JW. Human papillomavirus types 6 and 11 have antigenically distinct strongly immunogenic conformationally dependent neutralizing epitopes. *Virology* 1994;205:329-35.

40. Breitburd F, Kirnbauer R, Hubbert NL, *et al.* Immunization with virus like particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. *J Virol* 1995;69:3959-63.

41. Bell JA, Sunberg JP, Ghim SJ, Newsome J, Jenson AB, Schlegel R. A formalin-inactivated vaccine protects against mucosal papillomavirus infection: a canine model. *Pathobiology* 1994;62:194-8.

42. Rose RC, Bonnez W, Reichman RC, Garcea RL. Expression of human papillomavirus type 11 L1 protein in insect cells: in vivo and in vitro assembly of viruslike particles. *J Virol* 1993;67:1936-44.

43. Hagensee ME, Carter JJ, Wipf GC, Galloway DA. Immunization of mice with HPV vaccinia virus recombinants generates serum IgG, IgM, and mucosal IgA antibodies. *Virology* 1995;206:174-82.

44. Cason J, Khan SA, Best JM. Towards vaccines against human papillomavirus type 16 genital infections. *Vaccine* 1993;11:603-11.

45. Bosch FX, Manos MM, Muñoz N, Sherman M, Jansen AM, Peto J, *et al.* Prevalence of human papillomavirus in cervical cancer: a worldwide prospective. *J Natl Cancer Inst* 1995;87:796-802.

46. Bonnez W, Rin CD, Rose RC, Reichman RC. Use of human papillomavirus type 11 virions in an ELISA to detect specific antibodies in humans with condylomata acuminata. *J Gen Virol* 1991;72:1343-7.

47. Bonnez W, Kashima HK, Leventhal B, Mounts P, Rose RC, Reichman RC, Shah KV. Antibody response to human papillomavirus type 11 in children with juvenile-onset recurrent respiratory papillomatosis. *Virology* 1992;188:384-7.

48. Rose RC, Bonnez W, Da Rin C, McCance DJ, Reichman RC. Serological differentiation of human papillomavirus types 11, 16 and 18 using recombinant virus-like particles. *J Gen Virol* 1994;75:2445-9.

49. Chan SY, Delius H, Halpern AL, Bernard HU. Analysis of genomic sequences of 95 papillomavirus types: uniting typing, phylogeny, and taxonomy. *J Virol* 1995;69:3074-83.

50. Kirnbauer R, Hubbert NL, Wheeler CM, Becker TM, Lowy DR, Schiller JT. A virus-like particle enzyme-linked immunosorbent assay detects serum antibodies in a majority of women infected with human papillomavirus type 16. *J Natl Cancer Inst* 1994;86:494-9.

51. Christensen ND, Hopfl R, DiAngelo SL, *et al.* Assembled baculovirus-expressed human papillomavirus type 11 L1 capsid protein virus-like particles are recognized by neutralizing monoclonal antibodies and induce high titres of neutralizing antibodies. *J Gen Virol* 1994;75:2271-6.

52. Campo MS, Grindlay GJ, O'Neil BW, Chandrachud LM, McGarvie GM, Jarrett WF. Prophylactic and therapeutic vaccination against a mucosal papillomavirus. *J Gen Virol* 1993;74:945-53 [erratum published in *J Gen Virol* 1993; 74:2038]

53. Christensen ND, Kreider JW, Kan NC, DiAngelo SL. The open reading frame L2 of cottontail rabbit papillomavirus contains antibody-inducing neutralizing epitopes. *Virology* 1991;181:572-9.

54. Jarrett WFH, O'Neill BW, Gaukroger JM, Smith KT, Laird HM, Campo MS. Studies on vaccination against papillomaviruses: the immunity after infection and vaccination with bovine papillomaviruses of different types. *Vet Rec* 1990;126:473-5.

55. Jarrett WF, Smith KT, O'Neil BW,Gaukroger JM, Chandrachud LM, Grindlay J, *et al.* Studies on vaccination against papillomaviruses: prophylactic and therapeutic vaccination with recombinant structural proteins. *Virology* 1991;184:33-42.

56. Pilacinski WP, Glassman DL, Glassman KF, Reed DE, Lum MA, Marshall RF, *et al.* Immunization against bovine papillomavirus infection. In: Papillomaviruses: Ciba Foundation Symposium 120. Chichester: Wiley, 1986: 136-56.

57. Mallon RG, Wojciechowicz D, Defendi V. DNA binding activity of papillomavirus proteins. *J Virol* 1987;61:1655-60.

58. Carter JJ, Hagensee MB, Lee SK, McKnight B, Koutsky LA, Galloway DA. Use of HPV 1 capsids produced by recombinant vaccinia viruses in an ELISA to detect serum antibodies in people with foot warts. *Virology* 1994;199:284-91.

59. Carter JJ, Wipf GC, Hagensee ME, McKnight B, Habel LA, Lee S-K, *et al.* Use of human papillomavirus type 6 capsids to detect antibodies in people with genital warts. *J Infect Dis* 1995;172:11-8.

60. Heim K, Christensen ND, Hoepfl R, Wartisch B, Pinzger G, Zeimet A, *et al.* Serum IgG, IgM, and IgA reactivity to human papillomavirus types 11 and 6 virus-like particles in different genecologic patient groups. *J Infect Dis* 1995;172:395-402.

61. Wikstrom A, van Doornum GJ, Quint WG, Schiller JT, Dillner J. Identification of human papillomavirus seroconversions. *J Gen Virol* 1995;76:529-39.

62. Nonnenmacher B, Hubbert NL, Kirnbauer R, Shah KV Muñoz N, Bosch FX, *et al.* Serologic response to human papillomavirus type 16 virus-like particles in HPV16 DNA-positive invasive cervical cancer and cervical intrepithelial neoplasia grade III patients and controls from Colombia and Spain. *J Infec Dis* 1995;172:19-24.

63. Wikstrom A, van Doornum GJJ, Kirnbauer R, Quint WGV, Dillner J. Prospective study on the development of antibodies against human papillomavirus type 6 among patients with condyloma acuminata or new asymstomatic infection. *J Med Virol* 1995;46:368-74.

64. Andersson-Ellstrom A, Dillner J, Hagmar B, Schiller J, Forssman L. No serological evidence for non-sexual spread of HPV16. *Lancet* 1994;344:1435.

65. Dillner J, Wiklund F, Lenner P, Eklund C, Fredriksson-Shanazarian V, Schiller JT, *et al.* Antibodies against linear and conformational epitopes of human papillomavirus type 16 that independently associate with incident cervical cancer. *Int J Cancer* 1995;60:377-82.

66. Heino P, Eklund C, Fredriksson-Shanazarian V, Goldman S, Schiller JT, Dillner J. Association of serum immunoglobulin G antibodies against human papillomavirus type 16 capsids with anal epidermoid carcinoma. *J Natl Cancer Inst* 1995;87:437-40.

67. Schiffman MH. Epidemiology of cervical human papillomaviruses. In: zur Hausen H, ed. Human pathogenic papillomaviruses. Heidelberg: Springer-Verlag, 1994.

68. Park DS, Selvey LA, Kelsall SR, Frazer IH. Human papillomavirus type 16 E6, E7 and L1 and type 18 E7 proteins produced by recombinant baculoviruses. *J Virol Methods* 1993;45:303-18.

69. Le Cann P, Coursaget P, Iochmann S, Touze A. Self-assembly of human papillomavirus type 16 capsids by expression of the L1 protein in insect cells. *FEMS Microbiol Lett* 1994;117:269-74.

70. Zhou J, Sun XY, Davies H, Crawford L, Park D, Frazer IH. Definition of linear antigenic regions of the HPV16 L1 capsid protein using synthetic virion-like particles. *Virology* 1992;189:592-9.

Volume 6

PAPILLOMAVIRUS
REPORT

International literature on papillomaviruses

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Progress in the Development of HPV Vaccines

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Human papillomaviruses (HPVs) are the cause of common warts, plantar warts, and genital warts, as well as being associated with the majority of anogenital malignancies. These viruses cause substantial morbidity and mortality worldwide. In recent years, great progress has been made in gaining an understanding of the basic biology of HPV infection and in the development of immunoassays to detect HPV antibodies. Prophylactic and therapeutic phase I/II human vaccine trials are either under way or in the planning stage.
[Infect Med 14(7):555-556,559-564, 1997]

Key words: Human papillomavirus • DNA tumor virus • Vaccines • Cervical cancer • Genital warts • Immunoassays • Immunosuppression

Human papillomaviruses (HPVs) are associated with benign and malignant lesions of the anogenital tract[1,2] and are also responsible for common warts.[3] More than 70 different types of HPV have been identified (Table I). These HPVs show both tissue specificity and high or low malignant potential.[4] For example, DNA from HPV types 6 and 11 is commonly found in genital warts (condylomata acuminata) as well as in respiratory papillomas.[5,6] HPV types 16, 18, and other high-risk types (31, 33, 35, 45, 56; see

Table I) are found in anogenital malignancies[2]; HPV types 1 and 2 are found in common warts and plantar warts[3]; and HPV types 5 and 8 are associated with skin cancer in patients afflicted with the rare hereditary skin disease epidermodysplasia verruciformis.[7]

Scope of the Problem

Cutaneous plantar and palmar warts are ubiquitous, with the highest incidence seen in children and young adults. The prevalence of cutaneous warts decreases in adulthood, presumably due to the

development of host immunity.[8] The infections are usually benign and are rarely brought to the attention of health care providers.

Genital HPV infection is the most common sexually transmitted viral disease, with the prevalence ranging from 10%-50% (Fig. 1) in sexually active women.[1,9] The incidence in sexually active men has been less well studied but is thought to be similar to that seen in sexually active women. Because genital HPV infection is not a reportable disease, reliable estimates of its prevalence are difficult to obtain. The Centers for Disease Control and Prevention (CDC) estimates that since 1990 more than 1 million new cases of genital warts have been diagnosed in the US each year,[10] compared with fewer than 200,000 cases in 1966.[11]

HPV infection has been linked to cervical cancer and other anogenital tumors, such as penile, vulvar, vaginal, and anal cancers.[12] In women with cervical cancer, HPV DNA of high-risk type can be detected by polymerase chain reaction (PCR) in up to 90% of all cervical cancers. Many investigators propose that high-risk types of HPV are the direct cause of all cervical cancer.[2,13]

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Table I
HPV Types and Associated Disorders

HPV Type	Associated Disorder
HPV-1	Plantar and common warts
HPV-2	Plantar and common warts
HPV-4	Plantar and common warts
HPV-5	Skin cancer
HPV-6	Genital warts, respiratory papillomas
HPV-7	Common warts
HPV-8	Skin cancer
HPV-11	Genital warts, respiratory papillomas
HPV-16	Anogenital malignancies
HPV-18	Anogenital malignancies
HPV-31	Anogenital malignancies
HPV-33	Anogenital malignancies
HPV-35	Anogenital malignancies
HPV-45	Anogenital malignancies
HPV-56	Anogenital malignancies

Although the incidence of cervical cancer is decreasing in the US, it is the most common malignancy of women in developing countries, with about 500,000 new cases worldwide each year.[14]

Cervical cancer develops gradually over time, with precursor lesions or cervical intraepithelial neoplasia (CIN) usually occurring prior to invasive cancer.[15] The la-tency period between initial HPV infection and cancer may be more than 10 years.[16] These premalignant stages can be identified both clinically by speculum examination (Fig. 2) and in the laboratory by Papanicolaou (Pap) smear analysis.

Early detection of cervical cancer in the premalignant stages is the goal of routine cervical cancer prevention efforts (such as repeat speculum examination and Pap smear analysis). The decreased incidence of cervical cancer in the US is primarily due to routine screening of women with Pap smears.[17,18] However, not all women participate in routine screening. Furthermore, the cost of routine Pap smears on a national basis may exceed $10 million per year,[19] which makes this screening method implausible for many developing countries.

HPV infection and disease is more prevalent and severe in immunocompromised patients, particularly renal transplant patients[20] and individuals infected with HIV. Such immunocompromised individuals are at high risk for developing, at times, large disabling genital warts as well as premalignant anogenital lesions due to HPV.[20]

HIV-infected adults report higher incidences of HPV infection, as defined by the presence of HPV DNA, as well as higher incidences of CIN and anogenital condylomata. The anogenital condylomata observed are more difficult to treat in HIV-infected adults than in noninfected individuals.[21] Although women with HIV report higher incidences of CIN,[21] no significant increase in the incidence of cervical cancer has been noted in this population to date. However, as the life expectancy for HIV-infected women increases, cervical cancer in this group may become a significant health care problem.

Anal cancer is found predominately in homosexual men with or without concomitant HIV infection.[21,22] It is possible that prophylactic screening of the rectal mucosa with a Pap-like screening test is warranted in these patients and is currently under investigation.[23]

Treatment. Therapy for HPV in-
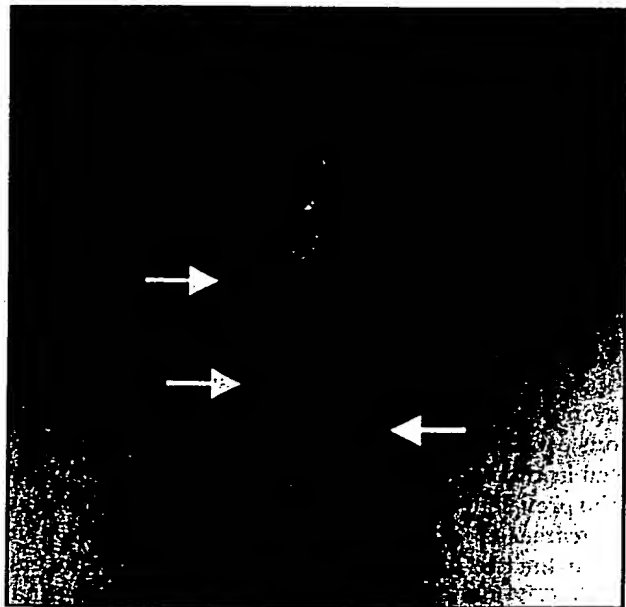
Figure 1. *Typical appearance of anogenital warts. (Courtesy of Dr. J. Torres, 1994.)*
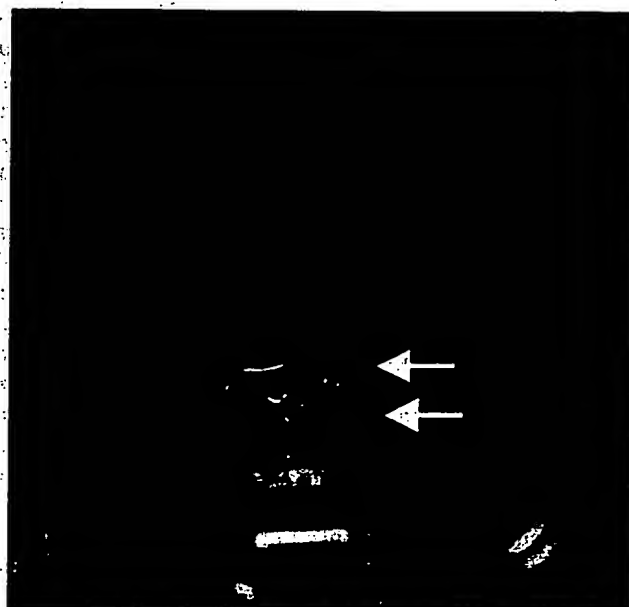
Figure 2. *Colposcopic view of cervical intraepithelial neoplasms type II and leukoplakia. (Courtesy of Dr. J. Torres, 1994.)*

fection consists chiefly of ablative approaches.[8] Genital warts are usually treated with liquid nitrogen, podophyllin, or laser ablation; premalignant cervical and other anogenital lesions are removed surgically. Severe cases of genital or respiratory papillomas respond to either intralesional or systemic administration of interferon alfa.[24-26] However, immunosuppressed patients may develop large anogenital warts that do not respond to the usual treatment modalities, and surgical excision may be difficult to perform. In addition, genital warts commonly recur in both immunocompetent and immunosuppressed individuals. Finally, no completely effective treatment currently exists for established cervical cancer, which has a mortality rate of 60%.[27]

Clearly, HPV causes significant disease, and treatment options are limited. Preventative screening by Pap smear is effective, but it is expensive and requires patients to return to health care providers for routine assessments. Development of both prophylactic and therapeutic vaccines for HPV infections is therefore essential.

The HPV Genome
HPVs are small (55 nm), nonenveloped, icosahedral, double-stranded DNA viruses containing approximately 8 kilobases of coding region. HPV is difficult to propagate routinely in the laboratory. This may be due to the regulation of HPV proteins by the differentiated state of the cell, with most gene expression and protein production occurring in terminally differentiated epithelial cells.

Early genes. The genome is divided into early (E) and late (L) regions (Fig. 3). Of the known early genes, E1 and E2 are involved in viral replication. The function of 2 open reading frames, E3 and E8, is not known (not shown in Fig. 3).

The function of E4 and E5 is not currently known; however, E4 may interact and disrupt the cell's cytoskeleton. In bovine papillomavirus (BPV) infection, E5 is a transforming protein that interacts with growth factor receptors. E5's role in HPV is not fully understood, but it is not required for transformation of the cell. E6 and E7 are responsible for the oncogenic potential of the high-risk HPVs (eg, HPV types 16 and 18). These genes are thought to cause cancer by their interaction with tumor suppressor/cell cycle control genes. Such interactions have been demonstrated with the p53 tumor suppressor gene[28] and the retinoblastoma (Rb) tumor suppressor gene.[29] This type of interaction leads to immortalization of a particular cell. Eventually, these cells acquire additional mutations that lead to malignant potential, namely invasion and metastasis. When this is observed, the E6 and E7 genes are usually intact, but the

rest of the HPV genes are not.[30] In some cancers, such as cervical and other anogenital cancers, the viral genome is integrated into the host cell's chromosomal DNA.[2]

Recent studies show that the presence of antibodies to the E7 gene of HPV type 16 portends a poor prognosis in cervical cancer patients.[31] A high tumor load is thought to be associated with a poor outcome and also seems to be required to generate the antibody response to E7. However, it is currently unclear whether antibodies to E7 can be used as a marker of recurrent or residual disease.

Late genes and VLPs. The late genes consist of the structural proteins L1 and L2. These proteins are found only in terminally differentiated cells.[32,33] The L1 protein is the major capsid protein and provides the main structure of the virus. The L2 protein's function is not known, but it may help encapsidate the viral genome. Recently, in vitro ex-
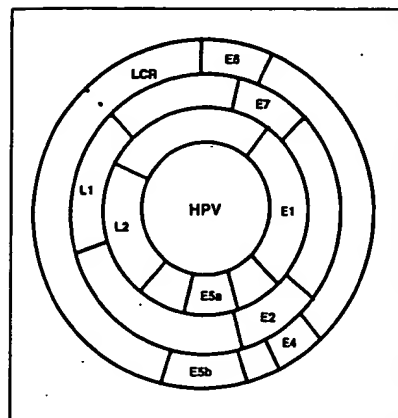
Figure 3. *Genomic organization of human papillomaviruses using HPV-11 as an example. Six early genes and 2 late genes are expressed from overlapping open reading frames.*
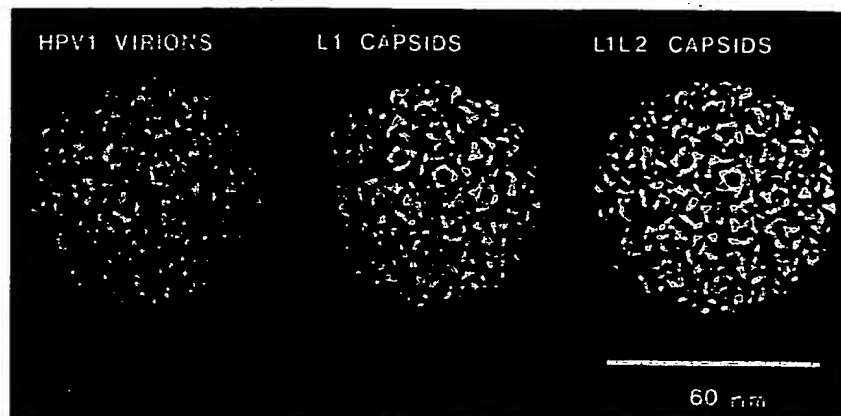

Figure 4. *Electron micrograph of surface-shaded representation of 3-dimensional reconstruction of HPV-1 isolated from foot warts (left), laboratory-produced capsids containing only L1 protein (middle), or L1 and L2 proteins (right). Reprinted with permission from J Virol (1994; 68:4503–4505), Copyright © 1994, American Society for Microbiology.*

pression of the L1 gene (using vaccinia virus, baculovirus, or yeast) has been shown to generate empty capsids or virus-like particles (VLPs) for many types of HPV.[34-37] The VLPs are structurally identical to virus isolated from warts, as demonstrated by high-resolution electron microscopy with 3-dimensional reconstruction (Fig. 4).[38]

VLPs are now being used as antigenic targets for the development of immunoassays for various HPV types.[39-43] The antibody response to HPV capsid proteins in serum correlates with the presence of HPV DNA and HPV-induced disease (genital warts or anogenital malignancy).[39,44] Furthermore, animal studies suggest that capsid antibodies have a neutralizing effect,[40] and neutralizing sera can be isolated from patients infected with HPV type 11.[45]

Vaccine Development

A number of the gene products of HPV are being considered for use as vaccines. For instance, a prophylactic vaccine is currently being proposed that would consist of VLPs containing the L1 protein alone or both the L1 and L2 proteins, because neutralizing antibodies against these capsid pro-

teins have been found in patients with HPV.[40,45]

Potential difficulties. One difficulty with developing a vaccine is determining which types of HPV to include. For example, HPV types 6 and 11 are responsible for approximately 90% of genital-wart cases, whereas HPV types 16 and 18 are found in 70%-80% of cervical cancers. A wide variety of other types account for the remainder of cases.[46]

Because cervical cancer takes many years to develop, a second difficulty is demonstrating the efficacy of a cervical cancer vaccine. It may be necessary to vaccinate young women before they become sexually active and to wait as long as 10 years before a reduction in the rate of cervical cancer is demonstrated. Alternatively, genital warts may be somewhat more amenable to study, since the disease occurs primarily in young adults.

In addition, for effective prophylaxis to occur, it may be necessary to produce antibody at the mucosal surfaces. Investigators are beginning to study the mucosal immune response to HPV infections and formulate vaccines to target mucosal surfaces. Recent studies have detected cervical IgA and IgG antibodies to HPV type 16 in patients

who are positive for HPV-16 DNA or have HPV-16–associated CIN.[47,48]

Therapeutic vaccine targets. There are many potential antigenic targets for a therapeutic vaccine. The replication proteins E1 and E2 may be required for establishment of a genital infection. The E4 and E5 transcripts are the most abundant, making them possible targets, although the function of these proteins is not known. E6 and E7 are being considered for a therapeutic cervical cancer vaccine since they are expressed during the late stages of disease. Finally, L1 and L2 are not always present in late stages of disease, making their use as potential therapeutic targets still plausible but with limitations. For therapeutic vaccination purposes, the patient population and the type of HPV infection are well defined, but selection of the antigen is difficult because the existing therapeutic vaccines lack efficacy.

Animal Models of HPV Infection

Currently, no experimental animal system is suitable for testing potential HPV vaccines. However, animal papillomaviruses can be utilized in assessing critical requirements for the development of human vaccines.

Table II
HPV Vaccines in Clinical Development

Company	Components	Type	Development Stage
Apollon	Plasmid DNA types unknown	Unknown	Preclinical
Cantab	Live recombinant vaccinia virus expressing E6 and E7 genes of HPV-16 and -18	Therapeutic	Phase I/II
Cantab	Recombinant protein of L2 and E7 from HPV-6	Therapeutic	Phase IIa
Chiron	Under development	Therapeutic	Preclinical
MedImmune	VLP (L1) of HPV-11	Prophylactic	Phase I
Merck	VLP (L1) of various HPV types	Prophylactic	Preclinical
Merck	Naked plasmid DNA, HPV types unknown	Unknown	Preclinical

Bovine model. There are multiple types of BPV, and this virus attacks skin in a fashion somewhat similar to that of human viruses.[49] BPV type 4 can progress to cancer, but the genome is lost in this process—a scenario that is not analogous to that in humans.[50] Prophylactic vaccination with purified BPV or with bacterial fusion proteins expressing the BPV L1 protein protects cows from subsequent challenge.[51-54] Use of purified BPV virions as the immunogen demonstrates cross-protection to other related BPV types, but not to all types.[51] Limited data on the bovine system show that both the L2 and E7 proteins are possible therapeutic vaccine candidates.[52,54]

Rabbit model. Studies with cottontail rabbit papillomavirus (CRPV) date back to Shope in 1937.[55] CRPV causes skin papillomas that can spontaneously regress or progress to squamous cell cancer. Analysis has shown that E1, E2, E6, E7, and L1 are all essential genes for CRPV infection and are thus targets for vaccine development. Immunization with intact virus, empty L1 capsids, and the L1 and L2 proteins expressed as bacterial fusion proteins can be protective.[56-59] In addition, direct DNA vaccination with the L1 gene is an effective prophylactic.[60] Studies with CRPV have in-

dicated that the E1 or E2 proteins could be effective therapeutic vaccine targets.[61,62]

Canine model. The newly discovered canine oral papillomavirus (COPV) may prove to be a better model of human disease, because it causes disease on a mucosal surface. Although this virus does not naturally undergo malignant progression, intramuscular injection of purified COPV does occasionally lead to squamous cell carcinoma.[49] Recent data show that only native empty capsids of COPV made in a baculovirus system are protective,[63] whereas denatured capsids afford no protection.

Rhesus monkey model. A rhesus monkey papillomavirus was isolated[64] from a lymph node metastasis from a monkey with penile cancer. This virus is similar to high-risk HPV types 16 and 18. Retrospective analysis indicated that this isolate may have caused premalignant lesions in female sexual partners of the infected male. However, owing to the expense of working with monkeys, extensive studies of this virus have not been undertaken. Nevertheless, the rhesus monkey model could serve to test potential human vaccines.

Other models. Heterologous animal model systems (ie, animals that

can accept human tissues without rejection) have been developed in order to better understand HPV. HPV-11 can be propagated in the laboratory by implantation under the renal capsule in a xenograft system using nude or severe combined immunodeficient (SCID) mice.[65,66] An analysis of the infectious cycle of HPV-11 using this system illustrated the generation of condylomata-like lesions in 10 to 12 weeks.[32] In this model, polyclonal rabbit, monoclonal mouse, and human serums directed against capsid antigens are protective against infection,[42,67,68] supporting the use of capsid proteins as prophylactic vaccines. Unfortunately, for reasons that are unclear, this model has not been effectively adapted for use with other genital types of HPV; thus the range of this protective effect remains undetermined. A mouse tumor rejection model showed that both HPV-16 E6 and E7 are tumor rejection genes.[69,70] Injection of mice with cells expressing HPV-16 E7 elicits an immune response that leads to the rejection of subsequently introduced E7-bearing tumor cells. Similar findings are seen for HPV-16 E6. This lends support to the proposed use of either E6 or E7 from high-risk HPV types as a therapeutic vaccine.

Editorial Comment: Treatment and Prevention of HPV Infection

Clinicians in primary care and virtually all specialties are frequently confronted with patients suffering from HPV infections or the malignancies that evolve from these infections. Prevention of infection requires avoidance of contact or use of barriers such as condoms, which inadequately cover infected cutaneous surfaces. We use dozens of treatment modalities, including liquid-nitrogen cryotherapy, bichloracetic acid, cantharidin, surgical excision, CO₂ laser surgery, electrodesiccation, vascular-lesion laser surgery, intralesional bleomycin, podophyllin, salicylic acid, contact immunotherapy, formaldehyde soaks, intralesional interferon, x-ray therapy, and even hypnosis.

As with most conditions, the length of the list of currently accepted treatment options is inversely proportional to the effectiveness of these regimens. Furthermore, problems with recurrence complicate the cases that are effectively treated. The cost to society to manage this problem is huge.

It is gratifying to see that progress is being made in developing vaccines to prevent and treat HPV-induced disease. Only after a safe and effective vaccine is available will we be able to reverse the HPV epidemic currently afflicting millions of our patients. A positive impact on the prevention and treatment of malignancies that evolve from these infections is also eagerly anticipated.

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Drugs Mentioned In This Article	
Interferon alfa	*Roferon-A, Intron A, Alferon-N*
Podophyllin (podophyllum resin)	*Pod-Ben, Podofin,* generic

The target population for therapeutic vaccines is better defined, but it is unclear which antigen to use. Over the next few years, additional human trials will be conducted for both treatment and prophylaxis of diseases caused by this ubiquitous group of viruses. □

Clinical Trials

Numerous pharmaceutical companies and independent investigators are planning or initiating phase I/II trials of prophylactic or therapeutic HPV vaccines in humans (Table II).[71] One method targets HPV types 6 and 11 genital wart infection with either a prophylactic approach using empty capsids or a therapeutic approach using E7 proteins with and without empty capsids as immunogens. The advantages of investigating genital wart vaccines first include the high prevalence of the disease and the reasonable time period required to show efficacy.

Development of a cervical cancer vaccine for HPV is directed primarily toward the E6 and E7 genes of high-risk types for use in patients with advanced disease. An initial phase I/II trial in England used the E6 and E7 gene products from HPV types 16 and 18 as immunogens expressed in vaccinia virus.[72] Eight patients with invasive cervical cancer (International Federation of Gynecology and Obstetrics stages Ib to IVb) were enrolled. The use of live vaccinia virus recombinants in these patients triggered no serious side effects. Only 1 patient developed a strong cytotoxic T-cell response to these HPV proteins after vaccination, but this same patient also had a long-term (>1 year) disease-free remission. This trial has stimulated further interest in developing similar vaccines for advanced HPV disease.

Conclusions and Future Directions

Vaccine development for HPV infection is quite exciting and is currently progressing rapidly, despite the lack of a good animal model and the difficulty in propagating the virus in the laboratory. The antigen of choice for prophylactic vaccines will most likely be the major capsid protein L1. Still unresolved are the questions of what HPV types to include in the vaccine and when to vaccinate.

References

1. Koustky LA, Galloway DA, Holmes KK: Epidemiology of genital human papillomavirus infection. Epidemiol Rev 10:122-163, 1988.
2. zur Hausen H: Human papillomaviruses in the pathogenesis of anogenital cancer. Virology 184:9-13, 1991.
3. Orth G, Favre M, Croissant O: Characterization of a new type of human papillomavirus that causes skin warts. J Virol 24:108-120, 1977.
4. de Villiers EM, Gissman L, zur Hausen H: Molecular cloning of viral DNA from human genital warts. J Virol 40:932-935, 1981.
5. Gissman L, Wolnik L, Ikenberg H, et al: Human papillomavirus types 6 and 11 DNA sequences in genital and laryngeal papillomas and in some cervical cancers. Proc Natl Acad Sci U S A 80:560-563, 1983.
6. Brown DR, Bryan JT, Cramer H, et al: Analysis of human papillomavirus types in exophytic condylomata acuminata by hybrid capture and Southern blot techniques. J Clin Micro 31:2667-2673, 1993.
7. Ostrow R, Bender M, Nimura M, et al: Human papillomavirus DNA in cutaneous primary and metastasized squamous cell carcinomas from patients with epidermodysplasia verruciformis. Proc Natl Acad Sci U S A 79:1634-1638, 1982.
8. Phelps, WC, Alexander, KA: Antiviral therapy for human papillomaviruses: Rationale and prospects. Ann Intern Med 123:368-382, 1995.
9. Nuovo GJ, Blanco JS, Leipzig S, et al: Human papillomavirus detection in cervical lesions nondiagnostic for cervical intraepithelial neoplasia: Correlation with Papanicolaou smear, colpolscopy, and occurrence of cervical intraepithelial neoplasia. Obstet Gyncol 75:1006-1011, 1990.
10. Diagnostic and therapeutic technology assessment: Human papillomavirus DNA testing in the management of cervical

neoplasia. JAMA 270:2975-2981, 1993.

11. Centers for Disease Control and Prevention: Condyloma acuminatum—United States, 1966-1981. MMWR 32:306-308, 1983.

12. zur Hausen H: Viruses in human cancer. Science 254:1167-1173, 1991.

13. Schiffman MH: Recent progress in defining the epidemiology of human papillomavirus infection and cervical neoplasia. J Natl Cancer Inst 84:394-398, 1992.

14. Munoz N, Bosch FX: Epidemiology of cervical cancer, in Munoz N, Bosch FX, Jensen OM (eds): Human Papillomaviruses and Cervical Cancer. Lyon, IARC Scientific Publications, 1989, pp 9-40.

15. Cason J, Khan SA, Best JM: Towards vaccines against human papillomavirus type-16 genital infections. Vaccine 11:603-611, 1993.

16. zur Hausen H: Intracellular surveillance of persisting viral infection: Human genital cancer results from deficient cellular control of papillomavirus gene expression. Lancet 2:489-491, 1986.

17. Cannistra SA, Niloff JM: Cancer of the uterine cervix. N Engl J Med 334:1030-1038, 1996.

18. Korn AP: Interpretation of abnormal Pap smears. Infect Med 13:405-406, 409-413, 1996.

19. Centers for Disease Control and Prevention: Results from the National Breast and Cervical Cancer Early Detection Program, October 31, 1991-September 30, 1993. MMWR 43:530-534, 1994.

20. Benton C, Shahidullah H, Hunter JA: Human papillomavirus in the immunosuppressed. Papillomavirus Report 3:23-26, 1992.

21. Palefksy J: Human papillomavirus infection among HIV-infected individuals. Hematol Oncol Clin North Am 5:357-370, 1991.

22. Daling JR, Weiss NS, Hislop TG, et al: Sexual practices, sexually transmitted diseases, and the incidence of anal cancer. N Engl J Med 317:973-977, 1987.

23. Kiviat NB, Critchlow CW, Holmes KK, et al: Association of anal dysplasia and human papillomavirus with immunosuppression and HIV infection among homosexual men. AIDS 7:43-49, 1993.

24. Reichman RC, Oakes D, Bonnez W, et al: Treatment of condyloma acuminatum with three different interferons administered intralesionally: A double-blind, placebo-controlled trial. Ann Intern Med 108:675-679, 1988.

25. Leventhal BG, Kashima HK, Mounts P, et al: Long-term response of recurrent respiratory papillomatosis to treatment with lymphoblastoid interferon alfa-n1. N Engl J Med 325:613-617, 1991.

26. Reichman RC, Oakes D, Bonnez W, et al: Treatment of condyloma acuminatum with three different interferon-alpha preparations administered parenterally: A double-blind, placebo-controlled trial. J Infect Dis 162:1270-1276, 1990.

27. Jordan M: Treatment of CIN by destruction, in Jordan JA, Sharp F, Singer A (eds): Preclinical Neoplasia of the Cervix. London, Royal College of Obstetricians and Gynaecologists, 1982, pp 185-186.

28. Scheffner M, Werness BA, Huibregtse JM, et al: The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 63:1129-1136, 1990.

29. Dyson N, Howley PM, Munger K, et al: The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science 243:934-937, 1989.

30. Schwarz E, Freese UK, Gissman L, et al: Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature (London) 314:111-114, 1985.

31. Baay MFD, Duk JM, Burger MPM, et al: Antibodies to human papillomavirus type 16 E7 related to clinicopathological data in patients with cervical carcinoma. J Clin Pathol 48:410-414, 1995.

32. Stoler MH, Whitbeck A, Wolinsky SM, et al: Infectious cycle of human papillomavirus type 11 in human foreskin xenografts in nude mice. J Virol 64:3310-3318, 1990.

33. Brown D, Fan L, Jones J, et al: Colocalization of human papillomavirus type 11 E1, E4 and L1 proteins in human foreskin implants grown in athymic mice. Virology 201:46-54, 1994.

34. Kirnbauer R, Booy F, Cheng N, et al: Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. Proc Natl Acad Sci U S A 89:12180-12184, 1992.

35. Hagensee ME, Yaegashi N, Galloway DA: Self-assembly of human papillomavirus type 1 capsids by expression of the L1 protein alone or by coexpression of the L1 and L2 capsid proteins. J Virol 67:315-322, 1993.

36. Rose RC, Bonnez W, Reichman RC, et al: Expression of human papillomavirus type 11 L1 protein in insect cells: In vivo and in vitro assembly of virus-like particles. J Virol 67:1936-1944, 1993.

37. Hoffman KJ, Cook JC, Joyce JG, et al: Sequence determination of human papillomavirus type 6a and assembly of virus-like particles in Saccharomyces cerevisiae. Virology 209:506-518, 1995.

38. Hagensee M, Olson N, Baker TS, et al: Three-dimensional structural analysis of vaccinia virus-produced HPV-1 capsids. J Virol 68: 4503-4505, 1994.

39. Carter JJ, Wipf, G, Hagensee ME, et al: Use of human papillomavirus type 6 capsids produced by recombinant vaccinia viruses in ELISA to detect antibodies in people with genital warts. J Infect Dis 172:11-18, 1995.

40. Rose RC, Reichman RC, Bonnez W: Human papillomavirus (HPV) type 11 recombinant virus-like particles induce the formation of neutralizing antibodies and detect HPV-specific antibodies in human sera. J Gen Virol 75:2075-2079, 1994.

41. Kirnbauer R, Hubbert NL, Wheeler CM, et al: A virus-like particle enzyme-linked immunosorbent assay detects serum antibodies in a majority of women infected with human papillomavirus type 16. J Natl Cancer Inst 86:494-499, 1994.

42. Nonnemacher B, Hubbert NL, Kirnbauer R, et al: Serologic response to human papillomavirus type 16 (HPV-16) virus-like particles in HPV 16 DNA-positive invasive cervical cancer and cervical intraepithelial neoplasia grade III patients and controls from Colombia and Spain. J Infect Dis 172:19-24, 1995.

43. Carter JJ, Koutsky LA, Wipf GC, et al: The natural history of human papillomavirus type 16 capsid antibodies among a cohort of university women. J Infect Dis 174:927-936, 1996.

44. Wideroff L, Schiffman MH, Hoover R, et al: Epidemiologic determinants of seroreactivity to human papillomavirus (HPV) type

16 virus-like particles in cervical HPV-16 DNA-positive and -negative women. J Infect Dis 174:937-943, 1996.

45. Christensen ND, Krieder JW, Shah KV, et al: Detection of human serum antibodies that neutralize infectious human papillomavirus type 11 virions. J Gen Virol 73:1261-1267, 1992.

46. Bosch FX, Manos MM, Munoz N, et al: Prevalence of human papillomavirus in cervical cancer: A worldwide perspective. J Natl Cancer Inst 87:796-802, 1995.

47. Wang Z, Hansson B, Forslund O, et al: Cervical mucus antibodies against human papillomavirus type 16, 18, and 33 capsids in relation to presence of viral DNA. J Clin Microbiol 34:3056-3062, 1996.

48. Hagensee ME, Koutsky L, Lee S, et al: Detection of cervical antibodies against HPV 16 capsids in a cohort of university women. In preparation.

49. Brandsma JL: Animal models for human papillomavirus vaccine development, in Lacey C (ed): Papillomavirus Review: Current Research on Papillomaviruses. Leeds, Leeds Medical Information, 1996, pp 69-78.

50. Gaukroger JM, Bradley A, Chandrachud L, et al: Interaction between bovine papillomavirus type 4 and cocarcinogens in the production of malignant tumours. J Gen Virol 74:2275-2280, 1993.

51. Pilacinski WP, Glassman DL, Glassman KF, et al: Immunization against bovine papillomavirus infection. Ciba Found Symp 120: 136-156, 1986.

52. Jarrett WFH, O'Neil BW, Gaukroger JM, et al: Studies on vaccination against papillomaviruses: The immunity after infection and vaccination with bovine papillomaviruses of different types. Vet Rec 126:473-475, 1990.

53. Jarrett WFH, Smith KT, O'Neil BW, et al: Studies on vaccination against papillomaviruses: Prophylactic and therapeutic vaccination with recombinant structural proteins. Virology 184:33-42, 1991.

54. Campo, MS, Gridlay GJ, O'Neill BW, et al: Prophylactic and therapeutic vaccination against a mucosal papillomavirus. J Gen Virol 74:945-953, 1993.

55. Shope, RE: Infectious papillomatosis of rabbits: With a note on the histology. J Exp Med 58:607-624, 1933.

56. Christensen ND, Krieder JW, Kan NC, et al: The open reading frame L2 of cottontail rabbit papillomavirus contains antibody-inducing neutralizing epitopes. Virology 181:572-579, 1991.

57. Lin Y, Borenstein LA, Selvakumar R, et al: Effective vaccination against papilloma development by immunization with L1 or L2 structural protein of cottontail rabbit papillomavirus. Virology 187:612-619, 1992.

58. Lin Y, Borenstein LA, Ahmed R, et al: Cottontail rabbit papillomavirus L1 protein-based vaccines: Protection is only achieved with a full-length, nondenatured product. J Virol 67:4154-4162, 1993.

59. Christensen ND, Reed CA, Cladel NM, et al: Immunization with viruslike particles induces long-term protection of rabbits against challenge with cottontail rabbit papillomavirus. J Virol 70:960-965, 1996.

60. Donnelly JJ, Martinez D, Jansen KU, et al: Protection against papillomavirus with a polynucleotide vaccine. J Infect Dis 713:314-320, 1996.

61. Selvakumar R, Ahmed R, Wettstein FO: Tumor regression is associated with a spe-

HPV Vaccines *continued*

cific immune response to the E2 protein of cottontail rabbit papillomavirus. Virology 208:298-302, 1995.

62. Selvakumar R, Borenstein LA, Lin Y, et al: Immunization with nonstructural proteins E1 and E2 of cottontail rabbit papillomavirus stimulates regression of virus-induced papillomas. J Virol 69:602-605, 1995.

63. Suzich JA, Ghim S, Palmer-Hill FJ, et al: Systemic immunization with papillomavirus L1 protein completely prevents the development of viral mucosal papillomas. Proc Natl Acad Sci U S A 92:11553-11557, 1995.

64. Ostrow R, McGlennen RC, Shaver MS, et al: A rhesus monkey model for sexual transmission of a papillomavirus isolated from a squamous cell carcinoma. Proc Natl Acad Sci U S A 87:8170-8174, 1990.

65. Krieder JW, Howett MK, Leure-Dupree AE, et al: Laboratory production in vivo of infectious human papillomavirus type 11. J Virol 61:590-593, 1987.

66. Bonnez W, Rose RC, Da Rin C, et al: Propagation of human papillomavirus type 11 in human xenografts using the severe combined immunodeficiency (SCID) mouse and comparison to the nude mouse model. Virology 197:455-458, 1993.

67. Christensen ND, Kreider JW: Antibody-mediated neutralization in vivo of infectious papillomaviruses. J Virol 64:3151-3156, 1990.

68. Christensen ND, Krieder JW, Cladel NM, et al: Monoclonal antibody-mediated neutralization of infectious human papillomavirus type 11. J Virol 64:5678-5681, 1990.

69. Chen L, Thomas EK, Hu S, et al: Human papillomavirus type 16 nucleoprotein E7 is a tumor rejection antigen. Proc Natl Acad Sci U S A 88:110-114, 1991.

70. Chen L, Mizuno MT, Singhal MC, et al: Induction of cytotoxic T lymphocytes specific for a syngeneic tumor expressing the E6 oncoprotein of human papillomavirus type 16. J Immunology 148:2617-2621, 1992.

71. Hanissian, J: Emerging HPV vaccines. Infect Med 14:266,273-275,330, 1997.

72. Borysiewicz LK, Flander A, Nimako M, et al: A recombinant vaccinia virus encoding human papillomavirus types 16 and 18, E6 and E7 proteins as immunotherapy for cervical cancer. Lancet 347:1523-1527, 1996.

Lyme

ease activist who made the FOIA request, states on the Web site, "The importance of these studies, if you live in an area local to one of the facilities that has a risk for Lyme disease, is that your doctor is not correct when he says, 'You could not possibly have Lyme disease, there is no Lyme disease in the area.'"

Additional Sites

Two additional sites deserve mention. McSweegan Lyme Disease Links is a useful set of links maintained by Edward McSweegan, PhD, an NIH health science administrator and former Lyme disease program officer. The University of Rhode Island's Tick Research Laboratory, a site that was mentioned in a previous "Medicine on the Net" column, also provides a wealth of useful information about Lyme disease.

Summary

As you can see, there is a great deal of information on the Internet related to Lyme disease for both patients and clinicians. The sites described in this article should provide a useful entrée into this subject. ☐

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Prospects for human papillomavirus vaccine developm nt: merging HPV vaccines

Jeffrey F. Hines[a], Shin-je Ghim[b] and A. Bennett Jenson[b]

This review concentrates on recent advances in human papillomavirus vaccine development. Strategies for prophylactic HPV subunit vaccines utilizing recombinantly synthesized, immunogenic virus-like particles are discussed. Therapeutic strategies focusing on the induction of cell-mediated immunity and gene manipulation for the treatment of established HPV-associated disease are also reviewed.

Curr Opin Infect Dis 11:57–61. © 1998 Rapid Science Ltd.

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Abbreviations

CTL	cytotoxic T lymphocyte
HAI	hemagglutination inhibition
HLA	human leukocyte antigen
HPV	human papillomavirus
VLP	virus-like particle

Introduction

Scientific advances have broadened our understanding of the molecular biology of human papillomavirus (HPV) infection. Papillomaviruses consist of a 55 nm, non-enveloped, icosahedral-shaped virion. The genome is organized as closed, circular, double-stranded DNA of approximately 8000 base pairs in length. Its genome can be divided into three functional regions. The early region is composed of eight open reading frames whose gene products are responsible for viral DNA replication, transcriptional control and cellular transformation [1]. E6 and E7 encode oncoproteins that bind and interfere with the function of the cellular tumor suppressor gene products, p53 and pRB, respectively. Through a series of differential phosphorylations, interactions with cyclin proteins and interactions with other cellular proteins, E6 and E7 promote cell-cycle progression [2–4]. The late region contains genes that encode two structural capsid proteins for the virion: the major capsid protein (L1) and the minor capsid protein (L2). The long control region contains the origin of DNA replication, promoter elements and transcriptional enhancer sequences [1,5]. Over 70 types of human papillomaviruses have been described. Over one-third of these are known to infect mucosal squamous epithelium. The association of the mucosotrophic HPV infection with cervical dysplasia and cervical carcinoma is well-established.

Anogenital infection with HPV is sexually transmitted. Prevalence data are dependent upon the population being investigated and the technique used to detect evidence of viral infection. In some populations, the prevalence exceeds 40% [6]. Human papillomaviruses are grouped as low-risk, intermediate-risk or high-risk according to each genotype's association with benign or malignant disease [7]. Low-risk genotypes, such as types 6 and 11, are commonly detected in condylomata and low-grade squamous intra-epithelial lesions and almost never in carcinoma. Conversely, high-risk genotypes such as 16, 18, 45 and 56 are detected in greater than 90% of invasive carcinomas of the cervix. The E6 and E7 genes of high-risk genotypes encode oncoproteins critical to the process of cellular immortalization in human keratinocytes [1]. These expressed oncoproteins in high-risk genotypes are crucial to cellular transformation and presumably tumorigenesis.

Better understanding of HPVs has fueled a search for innovative, non-traditional and novel approaches to the prevention and treatment of cervical dysplasia and cervical carcinoma. Recent studies have demonstrated that recombinantly synthesized, immunologically active forms of HPV capsids (virus-like particles, VLPs) can be prepared and

purified in quantity. Basic investigation and animal studies support the use of these VLPs as immunogens in the prevention of genital warts, cervical dysplasia and cervical carcinoma. Other investigations support the use of HPV E6 and E7 oncopeptides in the stimulation of cell-mediated immunity, enhanced viral peptide recognition and tumor rejection.

Recombinant HPV VLPs are highly antigenic, protective in animal models, lack potentially carcinogenic viral DNA, and are therefore ideal candidates for a multivalent prophylactic subunit vaccine against HPV infection. Immunization with HPV oncopeptides may be beneficial in cervical cancer regression, prevention and rejection. The development of techniques to manipulate and modify viral genes and the host genome has similarly opened exciting possibilities for more targeted therapy. These translational strategies may be important in reducing the incidence of cervical dysplasia and cervical carcinoma worldwide, particularly in developing countries (Figure 1).

Prophylactic vaccines

Prophylactic HPV subunit vaccine strategies ultimately act by inducing protective anti-HPV antibodies that prevent infection. The inability to propagate HPVs in tissue culture has historically hampered previous attempts to develop

Figure 1. Continuum of human papillomavirus (HPV)-associated disease and the role of novel therapies
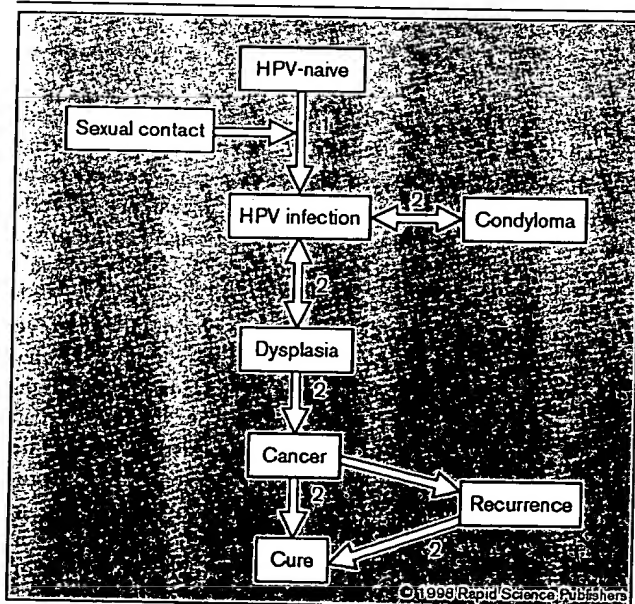
This schema summarizes effects of HPV infection in the development of genital warts, cervical dysplasia, and cervical carcinoma. Multivalent prophylactic HPV subunit vaccination (1) is likely to impact on the sequelae of HPV-associated anogenital disease. Therapeutic vaccines, immunotherapy and gene therapy (2) are likely to impact on the treatment of established genital warts, cervical dysplasia, and primary and recurrent cervical cancer.

conventional live-attenuated or killed vaccines. Exciting advances in recombinant molecular DNA technology now permit the expression of viral capsid proteins in a variety of systems to be used putatively as immunogens for strategies to prevent HPV infection. Papillomavirus conformational epitopes are critical to any discussion of HPV immunity and disease prophylaxis. Conformational epitopes are defined by the spatial arrangement of amino acids determined by the tertiary and quaternary folding and structure of a protein. The most important class of conformational epitopes are neutralizing epitopes found on the surface of native virions. Antibodies generated against neutralizing, conformational epitopes are sufficient to prevent infection *in vitro* and in animal models. Several authors now report on a variety of eukaryotic, yeast and other cell systems used to express recombinant viral protein and VLPs that mimic the conformation of native virions and react with conformation-dependent neutralizing antibodies [8–14].

Many investigations into HPV infection prevention focus on the expression of the major capsid protein of HPVs, with and without co-expression of L2, in eukaryotic cells that efficiently self-assemble into VLPs ultrastructurally indistinguishable from native virions by transmission electron microscopy. These empty capsids do not contain potentially oncogenic viral DNA. Importantly, these capsids induce high-titer, neutralizing antibodies in animal models. In addition to the humoral response elicited by VLPs, recent reports support a role of HPV-16 L1 VLPs in inducing T-cell responses as well [15•,16•]. It is likely that VLPs induce both cellular and humoral immunity.

Roden *et al.* recently reported on the use of VLPs in a hemagglutination inhibiton (HAI) assay to evaluate the serological relatedness of HPV types [17••]. Use of the HAI assay confirmed that cross-protection between highly homologous types is likely, but overall, most HPV genotypes represent separate serotypes. The same authors also describe a system for generating virion pseudotypes [18••]. Virion pseudotypes are VLPs composed of BPV-1 genome encapsidated by HPV self-assembled L1 protein (i.e. HPV-16). Such a strategy allows for efficient *in vitro* evaluation of neutralization of high-risk types, papillomavirus (PV) assembly, infectivity and serologic relatedness.

Virus-like particles are immunogenic when injected into animals. Infection by PVs specific to beagle dogs (COPV), cottontail rabbits (CRPV) and calves (BPV) can be prevented by immunization with PV VLPs followed by experimental challenge with native virus [19,20,21•]. These *in vitro* and animal data support a role for the development of human vaccine clinical trials.

There are, however, numerous challenges which must be addressed. It is likely that a VLP subunit vaccine might aim to prevent all sequelae of anogenital HPV infection, that is

genital condylomata, cervical dysplasia and cervical carcinoma. However, the latency to the appearance of genital warts, dysplasia and cervical carcinoma following infection varies from weeks to decades. Evaluation of the efficacy of such a vaccine may be impossible to determine if an endpoint of cervical carcinoma is chosen. Thus, human trials may focus on the more immediate endpoints of prevention of genital warts or dysplasia. Ideally, an HPV-naïve population should be the target population of study. Current epidemiological data and present sexual practices may narrow this HPV-naïve population to children and young adolescents. This may pose moral and ethical dilemmas for population selection. Ultimately, a cohort of sexually active women (and men) who are HPV-naïve will need to be randomized in a placebo-controlled, double-blinded trial. Sample size will be determined by an appropriate power analysis taking into consideration the latency of the endpoint chosen, the percentage decrease in the endpoint desired, disease prevalence and other factors. The composition of a multivalent subunit HPV VLP vaccine also depends upon the endpoint selected. If an endpoint to reduce the incidence of genital warts and cervical dysplasia is selected, the subunit VLP vaccine should contain predominantly low- and intermediate-risk serotypes. As more data are generated regarding serotype cross-relatedness, these multivalent vaccines can become streamlined. Conversely, if an endpoint of decreasing the incidence of cervical carcinoma is selected, a multivalent subunit vaccine that includes the high-risk types 16, 18, 45 and 56 should theoretically prevent 70–80% of cervical carcinomas. Issues relating to HPV variants and spontaneously occurring mutations may ultimately affect the serotypes selected [22••].

Therapeutic vaccines

Cellular immunity, particularly the T-cell system, appears to play a crucial role in modulating the effects of HPV infection and associated disease. This is evident from observations of immunosuppressed individuals who more often develop condylomata, cervical dysplasia and cervical carcinoma compared with immunocompetent individuals. Following HPV infection of epithelial cells, endogenously derived viral epitope peptides of E6 and E7 traffic through the endoplasmic reticulum and Golgi apparatus to be presented at the cell surface in association with class I MHC molecules. Subsequent recognition of viral epitopes to CD8+ cytotoxic T lymphocytes (CTLs) causes a cascade release of perforin and cytokines which mediate targeted cellular destruction. Additionally, viral epitope peptides processed by lysosomes and derived mainly from phagocytized or pinocytized exogenous viral antigen sources are presented at the cell surface in association with MHC class II molecules and are recognized by CD4+ T-cells. Wu and Kurman provide an excellent review of the cytokines which appear to contribute to cell-mediated immunity in patients with papillomavirus-associated neoplasms [23•]. Human papillomavirus E6 and E7 epitope peptides are thus useful targets in the development of translational immunotherapy strategies.

In vitro studies have successfully identified human leukocyte antigen (HLA)-specific, human CTL epitope peptides of E6 and E7 of highly oncogenic HPV types. Human CTLs induced against several of these E6 and E7 peptides will cause lysis of HLA-specific, HPV positive cervical carcinoma cell lines [24,25]. Use of these epitope peptides provides the basis for translational adoptive therapy strategies.

Adoptive cellular transfer consists of collecting cells involved in host defenses from a cervical carcinoma patient or a histocompatible donor. The cells are then grown and activated *ex vivo* by cytokines such as recombinant IL-2 and then transferred back to the cancer patient as therapy. The rationale behind this therapy is that controlled *ex vivo* propagation of immune cells will more consistently give rise to effective anti-tumor responses compared with immune responses generated in the host *in vivo*. In mouse models, some protection against HPV-16 E6 and E7-positive tumors is obtained with CTLs generated by immunization with HPV-16 E6 and E7 epitope peptides. Similar results have been reported for HPV-18 E6 and E7 epitope peptides [26,27].

Several clinical trials using this approach are ongoing and some results have recently been published. Borysiewicz *et al.* used a recombinant vaccinia virus expressing E6 and E7 epitope peptides of HPV-16 and HPV-18 in an open-label phase I/II trial in eight patients with advanced cervical carcinoma. They reported no significant toxicities. Each patient mounted an anti-vaccinia antibody response. Three of the eight patients developed HPV-specific antibody responses. However, HPV specific CTL responses were detected in one of three evaluable patients [28••]. Trials in the Netherlands, Australia and at the National Cancer Institute (USA) are actively accruing patients.

Similar challenges plague the development of these various strategies. Multiple HLA allelic determinants of MHC class I necessitate careful histocompatibility matching. Human papillomavirus-associated primary, metastatic and recurrent tumors frequently exhibit down-regulation of allelic expression of MHC class I molecules. Down-regulation may enable these carcinomas to escape cell-mediated immunity and negate the effects of a strategy designed to enhance immunity. If an endpoint of eradication of condylomata or cervical dysplasia is selected, spontaneous regression rates need to be considered. Again, sample size will be determined by an appropriate power analysis taking into consideration the latency of the endpoint chosen, the percentage decrease in the endpoint desired, disease prevalence and other factors.

G n therapy

The diverse and multiple techniques of transferring genetic material to a cell for potential therapeutic benefit comprise gene therapy. Viral vectors, mRNA and oligonucleotides have all been used, and recently reported experiences (see

below) with cervical carcinoma cell lines suggest that this may serve as a novel approach to targeted therapy.

Beer-Romero *et al.* reported that antisense targeting of E6-associated protein, which is required for E6-dependent degradation of p53, could be efficiently accomplished in HPV-infected cells and not in normal cells. Elevated levels of p53 could be detected [29*]. Modulation of p53 by eliminating a co-factor involved in its degradation holds promise as a therapeutic means of gene therapy. Hamada *et al.* utilized adenoviral transfer vectors to mediate transfer of wild-type p53 genes and E6/E7 antisense RNA into a human cervical carcinoma cell line. Induction of apoptosis and cell growth suppression was evident in cell lines transfected with wild-type p53 [30*]. HPV-16-positive cervical carcinoma cell lines transfected with antisense RNA transcripts of E6 and E7 genes of HPV-16 demonstrated greatly suppressed growth [31*]. Use of recombinant adenovirus as a gene delivery technique may therefore have great therapeutic potential for novel cancer gene therapy. Oligodeoxynucleotides can be specifically targeted against the HPV-16 E6 and E7 gene translational start regions and elicit cell growth inhibitory effects *in vitro* [32*]. These antiproliferative targets may have an active role in this strategy of antigene therapy.

Conclusions

Continued advances speed the molecular dissection of the HPV genome and enhance our understanding of HPV-mediated carcinogenesis. As the mechanisms of carcinogenesis become more fully unraveled and defined, new targets for novel pre-invasive and cancer therapies will be realized. These innovative therapies will likely be preventive, primary and adjuvant in nature. They are likely to be used in conjunction with more traditional therapies for pre-invasive disease and cancer. In an era of outcome-based, evidence-based and cost-effective endpoints in medicine, implementation of translational strategies of prophylactic vaccination for HPV-associated dysplasia and cervical carcinoma has the potential to significantly reduce the incidence of the more than 500 000 new cases of cervical carcinoma worldwide each year. Virus-like particles are the immunogen of choice for prophylactic HPV subunit vaccine clinical trials. Additional human trials are necessary to determine the roles of immunotherapy and gene therapy as treatment of cervical carcinoma.

R ferences and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:
* of special interest
** of outstanding interest

1 DiMaio D. Transforming activity of bovine and human papillomaviruses in cultured cells. *Adv Cancer Res* 1991; 56:133–159.

2 Sherr C. Mammalian G1 cyclins. *Cell* 1993; 73:1059–1065.

3 Weinberg R. The retinoblastoma protein and cell cycle control. *Cell* 1995; 81:323–330.

4 Lane D. Cancer: p53, guardian of the genome. *Nature* 1992; 358:15–16.

5 Broker T. Structure and genetic expression of papillomaviruses. *Obstet Gynecol Clin North Am* 1987; 14:329–348.

6 Bosch F, Manos M, Munoz N, Sherman M, Jansen A, Peto J. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. *J Natl Cancer Inst* 1995; 87:796–802.

7 Lorincz A, Reid R, Jenson A, Greenberg M, Lancaster W, Kurman R. Human papillomavirus infection of the cervix: relative risk of 15 common anogenital types. *Obstet Gynecol* 1992; 79:328–337.

8 Hines J, Ghim S, Christensen N, Kreider J, Barnes W, Schlegel R, Jenson A. Role of conformation epitopes expressed by human papillomavirus major capsid proteins in the serologic detection of infection and prophylactic vaccination. *Gynecol Oncol* 1994; 55: 13–20.

9 Hines J, Ghim S, Christensen N, Kreider J, Barnes W, Schlegel R, Jenson A. The expressed L1 proteins of HPV-1, HPV-6, and HPV-11 display type-specific epitopes with native conformation and reactivity with neutralizing and non-neutralizing antibodies. *Pathobiology* 1994; 62:165–171.

10 Kirnbauer R, Booy F, Cheng N, Lowy D, Schiller J. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc Natl Acad Sci USA* 1992; 89:12180–12184.

11 Sasagawa T, Pushko P, Steers G, Gschmeissner S, Hajibagheri M, Finch J, *et al.* Synthesis and assembly of virus-like particles of human papillomavirus type 6 and type 16 in fission yeast *Schizosaccharomyces pombe*. *Virology* 1995; 206:126–135.

12 Zhou J, Sun X, Stenzel D, Fraser I. Expression of vaccinia recombinant HPV-16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. *J Virol* 1991; 185:251–257.

13 Schiller J, Roden R. Papillomavirus-like particles. *Papillomavirus Rep* 1995; 6:121–128.

14 Nardelli-Haefliger D, Roden R, Benyacoub J, Sahli R, Kraehenbuhl J, Schiller J, *et al.* Human papillomavirus type 16 virus-like particles expressed in attenuated *Salmonella typhimurium* elicit mucosal and systemic neutralizing antibodies in mice. *Infect Immun* 1997; 65:3328–3336.

15 Dupuy C, Buzoni-Gatel D, Touze A, Cann P, Bout D, Coursaget P. Cell
* mediated immunity induced in mice by HPV 16 L1 virus-like particles. *Microb Pathogen* 1997; 22:219–225.
Data are presented which demonstrate that VLPs induce cellular immunity in addition to previously described humoral immunity.

16 Luxton J, Rose R, Coletart T, Wilson P, Shepherd P. Serological and T-
* helper cell responses to human papillomavirus type 16 L1 in women with cervical dysplasia or cervical carcinoma and in healthy controls. *J Gen Virol* 1997; 78:917–923.
Again, data are presented which demonstrate that VLPs induce cellular immunity in addition to previously described humoral immunity.

17 Roden R, Hubbert N, Kirnbauer R, Christensen N, Lowy D, Schiller J.
** Assessment of the serological relatedness of genital human papillomaviruses by hemagglutination inhibition. *J Virol* 1996; 70:3298–3301.
HAI is a simple assay that can monitor conformation-dependent and type-specific reactivity in individuals immunized with multiple HPV types.

18 Roden R, Greenstone H, Kirnbauer R, Booy F, Jessie J, Lowy D, Schiller J.
** In vitro generation and type-specific neutralization of a human papillomavirus type 16 virion pseudotype. *J Virol* 1996; 70:5875–5883.
HPV pseudotypes may facilitate the analysis of papillomavirus assembly, infectivity and serologic relatedness.

19 Suzich J, Ghim S, Palmer-Hill F, White W, Tamura J, Bell J, *et al.* Systemic immunization with papillomavirus L1 protein completely prevents the development of viral mucosal papillomas. *Proc Natl Acad Sci USA* 1995; 92:11553–11557.

20 Breitburd F, Kirnbauer R, Hubbert N, Nonenmacher B, Trin-Dinh-Desmarquet C, Orth G. Immunization with virus-like particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. *J Virol* 1995; 69:3959–3963.

21 Kirnbauer R, Chandrachud L, O'Neil B, Wagner E, Grindlay G,
•   Armstrong A, *et al.* Virus-like particles of bovine papillomavirus type 4 in
    prophylactic and therapeutic immunization. *Virology* 1996; 219:37–44.
Animal model demonstrating the effectiveness of VLPs as immunogens for
prophylactic vaccination.

22 Yamada T, Manos M, Peto J, Greer C, Munoz N, Bosch F, Wheeler C.
•• Human papillomavirus type 16 sequence variation in cervical cancers: a
    worldwide perspective. *J Virol* 1997; 71:2463–2472.
This study evaluates variation of DNA in HPV-16. Variants may prove to be
important for the determination of risk of dysplasia and cancer development
and the design of vaccine strategies.

23 Wu T, Kurman R. Analysis of cytokine profiles in patients with human
•   papillomavirus-associated neoplasms. *J Natl Cancer Inst* 1997; 89:185–
    187.
A comprehensive review of cytokines associated with HPV-mediated
diseases.

24 Feltkamp M, Smits H, Vierboom M. Vaccination with cytotoxic T
    lymphocyte epitope-containing peptide protects against a tumor
    induced by human papillomavirus type 16-transformed cells. *Eur J
    Immunol* 1993; 23:2242–2249.

25 Alexander M, Salgaller M, Celis E, Sette A, Barnes W, Rosenberg S,
    Steller M. Generation of tumor-specific cytolytic T lymphocytes from
    peripheral blood of cervical cancer patients by *in vitro* stimulation with a
    synthetic human papillomavirus type 16 E7 epitope. *Am J Obstet
    Gynecol* 1996; 175:1586–1593.

26 Krul M, Tijhaar E, Kleijne J, Van Loon A, Nievers M, Schipper H, *et al.*
    Induction of an antibody response in mice against human papillomavirus
    (HPV) type 16 after immunization with HPV recombinant *Salmonella*
    strains. *Cancer Immunol Immunother* 1996; 43:44–48.

27 Boursnell M, Rutherford E, Hickling J, Rollinson E, Munro A, Rolley N,
    et al. Construction and characterization of a recombinant vaccinia virus
    expressing human papillomavirus proteins for immunotherapy of cervical
    cancer. *Vaccine* 1996; 14:1485–1494.

28 Borysiewicz L, Fiander A, Nimako M, Wilkinson G, Westmoreland D,
•• Evans A, *et al.* A recombinant vaccinia virus encoding human
    papillomavirus types 16 and 18, E6 and E7 proteins as immunotherapy
    for cervical cancer. *Lancet* 1996; 347:1523–1527.
Phase I/II trial of E6/E7 proteins as immunotherapy in patients with advanced
cervical carcinoma. One patient of eight had a demonstrable effector CTL
response. Additional studies are warranted to evaluate this strategy further.

29 Beer-Romero P, Glass S, Rolfe M. Antisense targeting of E6AP elevates
•   p53 in HPV-infected cells but not in normal cells. *Oncogene* 1997;
    14:595–602.
Antisense targeting of E6AP increased p53 levels and inhibited cell growth.
Further investigation is warranted to evaluate the therapeutic potential of this
strategy of gene therapy.

30 Hamada K, Alemany R, Zhang W, Hittelman N, Lotan R, Roth J, Mitchell
•   M. Adenovirus-mediated transfer of a wild-type p53 gene and induction of
    apoptosis in cervical cancer. *Cancer Res* 1996; 56:3047–3054.
Transfection of cervical carcinoma cells with wild-type p53 via an adenoviral
vector caused increased p53 expression and suppressed cell growth.
Further investigation is warranted to evaluate the therapeutic potential of this
strategy of gene therapy.

31 Hamada K, Sakaue M, Alemany R, Zhang W, Horio Y, Roth J, Mitchell M.
•   Adenovirus-mediated transfer of HPV 16 E6/E7 antisense RNA to human
    cervical cancer cells. *Gynecol Oncol* 1996; 63:219–227.
Transfection of cervical carcinoma cells with E6/E7 antisense RNA via an
adenoviral vector suppressed cell growth. Further investigation is warranted
to evaluate the therapeutic potential of this strategy of gene therapy.

32 Madrigal M, Janicek M, Sevin B, Perras J, Estape R, Penalver M, Averette
•   H. *In vitro* antigene therapy targeting HPV-16 E6 and E7 in cervical
    carcinoma. *Gynecol Oncol* 1997; 64:18–25.
In vitro oligonucleotide targeting of start codon sequences of HPV-16 E6/E7
caused suppression of cell growth. Further investigation is warranted to
evaluate the therapeutic potential of this strategy of antigene therapy.

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